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Impact of *Bacillus amyloliquefaciens* Probiotic Strain H57 on the Intestinal Microbiota and Broiler Performance

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Abstract

The sub-therapeutic use of antibiotics as growth promoters in animal diets has been of concern and even been banned in some countries due to the emergence of antibiotic resistant bacteria, associated with human and animal disease. Probiotics are increasingly popular as an alternative to antibiotic growth promoters.

Bacillus amyloliquefaciens strain H57 (H57) is a spore forming aerobic bacterial strain that has been used as a probiotic with ruminants. This thesis examined H57 as a probiotic for broiler chickens; bird growth performance and feed efficiency along with its effects on the gastrointestinal microbiome and function were assessed.

Three broiler growth experiments were undertaken using diets with and without H57. The first experiment used a sorghum based diet, the second a wheat based diet and the third used three diets; i) sorghum based, ii) wheat based and iii) a sorghum and wheat blend (**chapter 3**). The effect of H57 on the intestinal microbial profile was studied using 16S rRNA gene sequencing of genomic DNA extracted from digesta samples from the ileum and caecum (**chapter 4**). The impact on potential caecal microbial function was studied using a shotgun metagenomic sequencing approach (**chapter 5**). The dynamics of H57 within intestinal content was measured by quantitative PCR (**chapter 6**).

Measurement of the population dynamics of H57 in the ileum and caecum by quantitative PCR indicated that there was no apparent multiplication of H57 cells in the gastrointestinal tract (GIT) and if spores did germinate, then vegetative growth was only sufficient to maintain numbers within the GIT.

Birds fed a sorghum based diet supplemented with H57 had improved body weight and feed conversion in both experiments 1 and 3. The response of H57 in wheat based diets varied between experiments; there was a positive effect of H57 in experiment 3 but not in experiment 2. In contrast, no improvement in bird performance was observed with birds fed a sorghum/wheat blend, with H57, in experiment 3. Notably, H57 had a positive effect on live weight gain only when control birds were underperforming, which presumably was ameliorated by H57.

H57 modified both the ileal and caecal microbial communities of the broilers. However, the effect of H57 on intestinal microbiota was not uniform across all experiments, diets (sorghum, wheat or mixed) or the age of the birds. There were significant differences in the intestinal microbial communities between different batches of chickens. Microbial community structure also varied

with the diet fed. Although there were several intestinal microbes with significantly different abundance between control and H57 birds, correlation analysis indicated that all of the microbes affected by H57 were not directly correlated to body weight gain, indicating that these microbes do not have a direct influence on growth. Moreover, this study showed that the caecal microbial functional potentiality of H57 treated and control birds, could differ even though the microbial population structure was similar. Therefore, it appears that the response to H57 was not mediated by changes in the microbial population *per se* but rather their functionality.

Several microbial functions in the caeca were significantly affected by feeding H57, both with wheat and sorghum based diets, as indicated by significantly different relative abundance of functional genes between H57 treated and control groups. Abundance of genes responsible for fermentation and energy metabolism (e.g. citric acid cycle and pentose phosphate pathway) were increased in the caeca of birds fed wheat based diets with H57, while the abundance of genes relating to microbial virulence (e.g. regulation of virulence, capsular and extracellular polysaccharide, biosynthesis of lipopolysaccharides, resistance to antibiotics) were significantly reduced in H57 fed birds fed the sorghum based diet. Increased fermentation rate as indicated by higher abundance of fermentation related genes in H57 treated birds may indicate a higher microbial digestion of non-starch polysaccharides, which are regarded as common anti-nutritional factor in wheat. Positive responses to H57 was only observed when there was growth depression in control birds **(chapter 3).** This was accompanied by modulation of intestinal microbial profiles **(chapter 4)** and fewer microbial virulence factors **(chapter 5)** in H57 treated chickens; leading to the presumption that H57 may modulate the intestinal microbiome and/or its function to reduce microbial virulence factor(s) responsible for the depression of growth in chickens. This could provide additional energy, otherwise used to maintain immune function, for increased live weight gain. The differences in the effects of H57 with different batches of chicks and different diet composition may have been due to inherent differences in the resident intestinal microbial populations in birds from different hatcheries as well as different batches from the same hatchery.

Thus, while *B. amyloliquefaciens* H57 appears to have significant potential for use as a probiotic in poultry production, its possible modes of action and production benefits remain to be fully elucidated.

Declaration by author

This thesis *is composed of my original work, and contains* no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

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Publications during candidature

Peer reviewed paper

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Conference abstracts

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Contributions by others to the thesis

- A/Prof. Athol Klieve, Prof. Wayne Bryden, A/Prof. Peter Dart, Ms Diane Ouwerkerk, Dr. Dagong Zhang, Dr. Xiuhua Li and Prof. Phil Hugenholtz all significantly contributed to the conception and design of this project.
- A/Prof Peter Dart and Mr. Ben Schofield assisted in the fermentation of H57 and preparation of H57 inoculum.
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Research Involving Human or Animal Subjects

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A copy of the ethics approval letter is included in the Appendix 4.

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Bacillus amyloliquefaciens H57, probiotics, poultry, microbial community profiling, metagenomics

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Table of Contents

List of Figures

List of Tables

List of Abbreviations

ADFI	Average Daily Feed Intake
AGP	Antibiotic Growth Promoters
AID	Apparent Ileal Digestibility
ANOVA	Analysis of Variance
BCO	Bacterial Chondronecrosis With Osteomyelitis
BLAST	Basic Local Alignment Search Tool
BW	Body Weight
BWG	Body Weight Gain
CD3	Cluster of Differentiation
CP	Crude Protein
DCA	Detrended Correspondence Analysis
DIAMOND	Double Index Alignment of Next-Generation Sequencing Data
DM	Dry Matter
EDTA	Ethylenediaminetetraacetic Acid
EFSA	European Food Safety Authority
EU	European Union
FAO	Food and Agriculture Organization of the United Nations
FCR	Feed Conversion Efficiency
GE	Gross Energy
GIT	Gastrointestinal Tract
GRAS	Generally Recognized as Safe
H57	*Bacillus Amyloliquefaciens* Strain H57
HUS	Haemolitic Uremic Syndrome
IEL	Intestinal Intraepithelial Lymphocytes
IFN	Interferon
Ig	Immunoglobulin
IL	Ilterleukin
KEGG	Kyoto Encyclopedia of Genes and Genomes
KEGGREST	Client-Side REST Access to KEGG
KO	Kegg Orthology
LAB	Lactic Acid Bacteria
LEE	Locus of Enterocyte Effacement
LPS	Lipopolysaccharides

MG-RAST	Rapid Annotations Using Subsystems Technology of Metagenomics
NE	Necrotic Enteritis
OTUs	Operational Taxonomic Units
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
PD	Phylogenetic Diversity
PERMANOVA	Permutational Multivariate Analysis af Variance
PGA	Polyglutamic Acid
pgsB	Poly-C-Glutamic Acid Synthase
QASP	Queensland Animal Science Precinct
QIIME	Quantitative Insights Into Microbial Ecology
qPCR	Quantitative PCR
RBB+C	Repeated Bead Beating Plus Column
SBM	Soybean Meal
SBS	Sequencing By Synthesis
SCFA(s)	Short Chain Fatty Acid(S)
SEM	Standard Error of Mean
SPRI	Solid-Phase Reversible Immobilization
STEC	Shiga-Toxin Producing *E. Coli*
TCR	T Cell Receptor
TE	Tris-EDTA
TGF	Transforming Growth Factor
TNF	Tumour Necrosis Factor
UQ	University of Queensland
USFDA	United States Food and Drug Administration
WHO	World Health Organization

Chapter 1 General introduction

1.1 Background

The world's population is expected to reach more than 9 billion by 2050, imposing food security challenges particularly for developing countries. Moreover, economic growth has increased the demand for livestock products putting pressure on the livestock sector to produce more with limited resources. Nevertheless, the livestock sector is one of the fastest growing agricultural sectors contributing about 40 % of the global value of agricultural production (Bruinsma, 2003), supporting the livelihoods and food security of almost 1.3 billion people.

Livestock provide a major source of disposable income for disadvantaged and marginal populations in developing countries and a major entry point to fight against rural poverty (Randolph et al., 2007, Smith et al., 2013). In addition to being a good source of income and nutrition, livestock provide draft power and manure for use as fuel and fertilizer. Also livestock enterprises can offer inflation-proof animal assets for insurance and financing (Ehui et al., 1998, Sansoucy et al., 1995). Intensive production systems are playing an increasingly important role in the livestock sector worldwide. Poultry are the cheapest source of animal protein significantly contributing to supplying the growing demand for animal food products around the world. The global poultry industry is growing faster than other agricultural commodities making it the second largest source of meat after pork.

Despite the benefits to many of increased livestock production, this has created two major public health issues. Firstly, sub-therapeutic use of antibiotics as growth promoters in animal feed has evoked widespread concern and their use banned in many countries, including the European Union (EU), due to the potential to develop antibiotic resistance in microbial populations associated with human and animal diseases. Secondly, some of the food borne zoonotic diseases like salmonellosis, campylobacteriosis and pathogenic *Escherichia coli* infection, among others are serious public health concerns around the world and can cause serious economic loss.

Probiotics (or direct fed microbials) are becoming increasingly popular as one of the alternatives to Antibiotic Growth Promoters (AGP). The most important objectives for using probiotics in animal feed are to maintain and improve the performance (productivity and growth) of the animal and prevent and control enteric pathogens. In the context of the growing concern with the sub-therapeutic use of AGP in animal feed and greater appreciation of the role of the microbial ecology of the gastrointestinal tract (GIT) in determining animal productivity, increasing numbers of probiotic products are being developed and used in animal nutrition.

1.2 Research problem

Sub-therapeutic use of antibiotics as growth promoters in animal feed has received widespread concern and even been banned from use in many countries. There is therefore a urgent need to fine feed additives to fill the void left by the reduction in the use of AGPs; probiotics have great potential in this regard. Two of the most important objectives for using probiotics in poultry feed are to promote productivity and control enteric pathogens.

Recently, there have been many studies to evaluate the performance of probiotics to improve the growth rate in poultry/broilers and to control or prevent of enteric diseases like salmonellosis, campylobacteriosis, necrotic enteritis etc. Although probiotics have been found to be beneficial in poultry production, the results are highly variable and there is limited information about their mode of action and effects on gastrointestinal microbial ecology. There is limited knowledge about the function of microbes occurring in the GIT. Currently there is no single probiotic strain which is beneficial across different production systems and which addresses all of the issues raised above.

Researchers from the University of Queensland (UQ) have isolated a novel strain of *Bacillus amyloliquefaciens* strain H57 (H57) from a lucerne plant at the UQ Gatton Campus. The strain has been shown to prevent mould development on hay. The strain was used in the commercial product HayRite. and studies have demonstrated benefits to animal agriculture, in terms of improved nitrogen retention and therefore reduced feed protein wastage in ewes fed hay treated with H57 (Brown and Dart, 2003). The bacterial strain was effective in enhancing the performance of sheep (Le et al., 2017).

1.3 Objectives of the PhD

There have been no studies using *B. amyloliquefaciens* strain H57 in nonruminant/monogastric animals. The current studies have been designed with the major objectives of assessing the effect of this bacterium in poultry and studying the effects of this probiotic on the function and profile of the gastrointestinal microbiota. It was also hoped that these studies would assist in the elucidation of the mode of action of this probiotic bacterium. Specific objectives were;

1. To evaluate the effects of *B. amyloliquefaciens* H57 on performance of poultry.
2. To study the effects of *B. amyloliquefaciens* H57 on the intestinal microbial population of poultry.

3. To study the effects of *B. amyloliquefaciens* H57 on the gastrointestinal microbial function in poultry.

To achieve these objectives, the candidate probiotic *B. amyloliquefaciens H57* has been produced in the fermentation facility established on the Gatton Campus of the University of Queensland. The bacterial spore produced were mixed in poultry feed and fed to broilers and bird productivity measured. Microbial profiling of the ileum and caecum of chicken was determined by 16s rRNA sequencing of the genomic DNA extracted from the intestinal contents. Metagenomic shotgun sequencing was used to study the microbial function in the caecum.

1.4 Significance of the project

Although the candidate probiotic *B. amyloliquefaciens* strain H57 has been found to be effective in ruminants, its efficacy has not been tested in monogastrics. Therefore, its efficacy has to be tested in a range of production scenarios, if this probiotic is to be developed further and is to be used in commercial poultry production. If the tested probiotic is found effective, it could be a candidate to replace antibiotic growth promoters in poultry feed which could reduce the public health hazard associated with the use of antibiotics as growth promoters in feed.

Chapter 2 Literature review[†]

2.1 Probiotics: Definition and classification

2.1.1 Definition

The term 'probiotics' was first used by Lilly and Stillwell (1965) to designate unknown growth promoting substances produced by a ciliate protozoan which stimulated the growth of another ciliate. The term now covers a much broader group of organisms. Parker (1974) defined probiotics as "organisms and substances which contribute to intestinal microbial balance" thus including both living organisms and non-living substances. Fuller (1989) was critical of the inclusion of the word 'substances' and redefined probiotics as "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance".

The joint Food and Agriculture Organization of the United Nations (FAO) and World Health Organization (WHO) working group defined probiotics as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO and WHO, 2001). This definition is widely accepted and adopted by the International Scientific Association for Probiotics and Prebiotics (Hill et al., 2014).

The FAO and WHO definition of probiotics as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" is the most widely accepted.

2.2 Microorganisms used in probiotics

Many commercial products use multi-strain probiotics although the benefits of using more than one strain or species in a single product has not been clearly established (Zhao et al., 2013). Microorganisms that have been used as probiotics in animal feed are listed in Appendix 1 table S1.

2.3 Mode of probiotic action

Although probiotics are found beneficial to improve production and growth in poultry, their mode of action is not always clear (Ajuwon, 2015). Different probiotics exert their effects through different mechanisms not fully understood and presumed to be due to their action either in the

[†] An extensive literature review with wider scope covering impacts of probiotics in poultry, pig and ruminants; and safety and regulation of probiotics has been done during candidature of this PhD and published by Food and Agriculture Organization of the United Nations (http://www.fao.org/3/a-i5933e.pdf). Remaining sections of literature review is given in Appendix 1.

gastrointestinal lumen or the wall of the GIT. Although probiotics are being promoted as a substitute for AGP, the mechanism of action of these feed additives appears to be different (Fajardo et al., 2012).

Probiotics help to prevent and control gastrointestinal pathogens and/or improve the performance and productivity of production animals through different mechanisms. Closely related strains may differ in their mode of action (Roselli et al., 2007, Fioramonti et al., 2003, Lodemann, 2010). There are increasing number of spore forming bacterial strains to be used as probiotics. A small proportion of ingested spores is believed to germinate in the intestine of animals (Casula and Cutting, 2002, Tam et al., 2006). However, it is not clear whether the germinated spores or the spores in its ingested form exert beneficial effects on the host. Major mechanisms of action proposed for probiotics are as follows.

2.3.1 Modification of the microbial population of the GIT: promoting favourable GIT microflora

Maintaining the gut health in animals, particularly in the context of AGP being gradually phased out, through the manipulation of diet is crucial to maintain/improve the performance of production animals (Choct, 2009). One of the major determinants of a healthy GIT is the composition of the microbial population. Probiotics can change the microbial population dynamics in the GIT eventually creating a more favourable microbial population due to a shift in the balance of beneficial and harmful microbes (Mountzouris et al., 2007, Mountzouris et al., 2009, An et al., 2008). Healthy microbial populations in the GIT are often associated with enhanced animal performance, reflecting more efficient digestion and improved immunity (Hung et al., 2012, Niba et al., 2009). The reduction in pathogenic microorganisms in the GIT may be attributable to the production of antimicrobial substances such as bacteriocins (Shim et al., 2012) and adhesion of the probiotic microbes to the intestinal epithelium, thereby excluding pathogens competitively or by inducing immune system response.

The most common modulation of the GIT microflora by probiotics (for example in chickens) is the increase in the populations of *Lactobacillus* and *Bifidobacteria* (Shim et al., 2012, Mountzouris et al., 2010, Mookiah et al., 2014, Abdelqader et al., 2013, Hung et al., 2012, Yang et al., 2012a, Zhang et al., 2011, Zhang et al., 2014a, Cao et al., 2013, Vahjen et al., 2002, Khaksar et al., 2012, Landy and Kavyani, 2013) while populations of coliforms particularly *Escherichia coli* (Shim et al., 2012, Mountzouris et al., 2010, Mookiah et al., 2014, Abdelqader et al., 2013, Hung et al., 2012, Yang et al., 2012a, Samli et al., 2010, Zhang et al., 2014b, Cao et al., 2013, Khaksar et al., 2012,

Landy and Kavyani, 2013) and *Clostridium* spp. (Shim et al., 2012, Abdelqader et al., 2013, Yang et al., 2012a, Cao et al., 2013) decrease. This pattern of modification of the GIT microflora occurs with all the common types of bacteria used as probiotics, such as lactic acid bacteria (LAB) (Mountzouris et al., 2010, Mookiah et al., 2014, Cao et al., 2013), spore forming bacteria (*Bacillus* spp.) (Shim et al., 2012, Abdelqader et al., 2013) and clostridial bacteria (*C. butyricum*) (Yang et al., 2012b, Zhang et al., 2011), and with both Gram positive and Gram negative strains (Hashemzadeh et al., 2013). In contrast, dietary supplementation of broiler diet with a commercial probiotic containing *S. cerevisiae* did not affect total aerobic microbes, lactose faecal coliforms, *Lactobacillus*, and *E. coli* in the content of all intestinal sections (duodenum, jejunum, ileum and caeca) at day 21 (Abdel-Raheem et al., 2012). At day 42 only the population of *Lactobacillus* in the duodenum was increased significantly without change in the population of all other measured microbes (as mentioned above) in all intestinal sections. However, the probiotic induced body weight increase of 9%, feed intake by 3% and FCR by 6%.

Lactobacilli and bifidobacteria produce proteins or polypeptide bacteriocins which reduce the growth of closely related bacterial species (Kawai et al., 2004, Yildirim and Johnson, 1998), which may reduce the number of harmful microorganisms in the GIT.

Lactobacillus adheres to the ileal epithelial cells of chickens (Jin et al., 1996). This may competitively exclude pathogenic microorganisms from the GIT (Mookiah et al., 2014). In addition, these bacteria produce short chain fatty acids (SCFA) such as acetic and lactic acid which can inhibit harmful microbes in the GIT (Jin et al., 1996, Watkins et al., 1982, Mookiah et al., 2014).

Probiotics may increase the population of beneficial microorganisms including lactobacilli and bifidobacteria, which then inhibit growth of harmful microorganisms by producing inhibiting substances (bacteriocins and/or organic acids) and by competitive exclusion.

However, because only a small proportion of the microbial flora in the GIT can be cultured, modern DNA sequencing methods are required to delineate the effects of probiotics on the animals GIT microbiome. In a probiotic dose response study, Mountzouris et al. (2010) showed that improvement in the growth rate of chickens occurred without a significant change in the populations of microbes in the GIT assessed using culture based techniques. Inclusion of the multi-strain commercial probiotic (PoultryStar ME) in poultry feed at 10^8 cfu/kg enhanced the growth rate of broiler chickens without an observable effect on caecal microflora composition. Increasing the concentration of the probiotic in feed to 10^9 cfu/kg, however, altered the caecal microbial populations reducing coliforms.

Two important points about the effects of probiotics in gastrointestinal microbial ecology are: i) There appear to be species specific effects of probiotics on GIT microflora and ii) Traditional culture-based techniques applied in most of the studies are not able to adequately reflect the actual GIT microbial population. As traditional culture-based techniques are extremely limited in their ability to decipher changes in microbial ecosystems, the application of modern molecular identification and sequencing techniques are required to provide insight into the effects of probiotics on the GIT microbiota.

2.3.2 Increase in digestion and absorption of nutrients

Improvements in productivity of animals due to probiotics can be associated with an increase in digestion and absorption of nutrients. The response in broiler chickens to dietary supplementation of *L. bulgaricus* varied with level of probiotic provided. At the rate of $2x10^6$ cfu/g there was no significant effect on digestibility of crude protein or fat, but at $6x10^6$ cfu/g and $8x10^6$ cfu/g there was a significant increase ranging from 7-11% for protein and 6.5 -13.4% for fat with 7.9-11.7% increase in weight gain (Apata, 2008). In another study, although supplementation of broiler diet with the commercial probiotic (AgiPro A100) increased digestibility of dry matter (DM) by 12.4% at day 42 (Li et al., 2008) weight gain, average daily gain, feed intake and FCR were not significantly affected. Similarly, probiotics increased the apparent ileal digestibility of essential amino acids with 5% improvement in body weight gain (Zhang and Kim, 2014) and improved the bioavailability of calcium in chicken (Chawla et al., 2013).

Increased digestibility of nutrients in diet may be due to increased enzyme activity in the intestine due to probiotics. *Lactobacillus* probiotics altered the digestive enzyme activity in the GIT of poultry and pigs. Amylase activity in the small intestine of poultry increased by 42% in response to *L. acidophilus* supplied at the rate of $2x10^6$ cfu/g of corn-soybean based diet (Jin et al., 2000). However, there was no change in proteolytic and lipolytic activity. This improvement in amylase activity was associated with a 4.6% increase in body weight gain and 5% improvement in feed use efficiency. Similarly, sucrose, lactase and amylase but not peptidase activity in the small intestine of pre-weaned pigs increased in response to a commercial probiotic (Probios) containing *L. plantarum, L. acidophilus, L. casei and E. faecium* (Collington et al., 1990).

Spore forming bacteria, like *Bacillus amyloliquefaciens*, produce extracellular enzymes including α-amylase, cellulase, proteases and metalloproteases (Gould et al., 1975, Lee et al., 2008, Gangadharan et al., 2008), which could increase nutrient digestion.

Increased enzyme activity in the gastrointestinal tract of animals supplemented with probiotics could be due to either production of enzyme by the probiotic itself or induced change in the microbial population and thence enzyme production.

Probiotics increased the height of intestinal villi and villus height: crypt ratio in poultry (see section 7.1.5) (Afsharmanesh and Sadaghi, 2014, Jayaraman et al., 2013, Biloni et al., 2013), thus increasing the surface area for nutrient absorption.

2.3.3 Production of antimicrobial substances

Some probiotics produce antimicrobial substances which may inhibit growth of pathogenic microorganisms in the intestine.

Many bacterial species including lactic acid bacteria (LAB) (Flynn et al., 2002, Nes et al., 1996, Klaenhammer, 1988), bifidobacteria (Cheikhyoussef et al., 2008) and bacillus (Hyronimus et al., 1998, Le Marrec et al., 2000) can produce several types of thermostable bacteriocins (Cotter et al., 2005) which have antimicrobial activity against a range of potential pathogens of animals including *Bacillus, Staphylococcus, Enterococcus, Listeria,* and *Salmonella* species (Rea et al., 2007, Flynn et al., 2002, Corr et al., 2007). Corr and colleagues (2007) demonstrated that the probiotic *L. salivarius* strain UCC118 produced a broad spectrum bacteriocin, Abp118, which protected mice against pathogenic *Listeria monocytogenes*. A mutant of the same probiotic unable to produce bacteriocins did not protect the mice confirming bacteriocins were the active agent.

Bacteriocin produced by LAB (for example Nisin) inhibits the growth of pathogenic microorganisms by inhibiting cell wall synthesis with the formation of pores in the bacterial surface (Hassan et al., 2012, Wiedemann et al., 2001). To achieve this, the bacteriocin binds the cell wall precursor, lipid II, forming a complex which can form a pore in the bacterial cell membrane leading to the death of the bacterium (Bierbaum and Sahl, 2009, Wiedemann et al., 2001).

Many probiotic bacteria, especially LAB producing SCFA, particularly lactic and acetic acids, can inhibit pathogenic bacteria (Commane et al., 2005, Fayol-Messaoudi et al., 2005). SCFA reduce the pH in microenvironments within the intestinal lumen and can then be taken up by GIT microbes in broiler chickens reducing their intracellular pH to a lethal level for some bacteria (Daskiran et al., 2012).

Probiotic bacteria produce other antimicrobial compounds which may inhibit harmful microbes in the GIT. Brashears et al. (1998) found that *Lactobacillus lactis* strains when inoculated in refrigerated raw chicken meat inoculated with *E. coli* 0157: H7 inhibits the growth of *E. coli* 0157:

H7 due to production of hydrogen peroxide. Does *Lactobacillus* produce hydrogen peroxide in the gastrointestinal environment? *B. subtilis* PB6, a bacterial strain isolated from the GIT of chickens produces a heat stable anticlostridial factor, which inhibited *Clostridium perfringens*, the causative agent of necrotic enteritis in poultry, *in vitro* as well as *Clostridium difficile*, *Streptococcus pneumoniae*, *Campylobacter jejuni*, and *Campylobacter coli* (Teo and Tan, 2005). Similarly, *B. amyloliquefaciens* a probiotic which improved performance of broiler chickens (Lei et al., 2015, Ahmed et al., 2014) produces several antimicrobial cyclic lipopeptide compounds (e.g. surfactin, fengycin, bacillomycin D, iturin A) (Sun et al., 2006, Ongena and Jacques, 2008, Chen et al., 2009, Arrebola et al., 2010) and polyketides (e.g. macrolactin, difficidin, bacillaene, chlorotetaine) (Chen et al., 2009, Rapp et al., 1988) which antagonise the growth of plant pathogens (Chen et al., 2009).

2.3.4 Alteration in gene expression in pathogenic microorganisms

Bacteria communicate cell to cell through the secretion of chemical signals called autoinducers, that affect the behaviour of bacteria (Miller and Bassler, 2001, Waters and Bassler, 2005). This process of bacterial communication, called quorum sensing, is also used for communication between bacteria and their host (Hughes and Sperandio, 2008).

Probiotics may affect quorum sensing in pathogenic bacteria thus influencing their pathogenicity. Extracellular secretion of a chemical signal (autoinducer-2) by human enterohaemorrhagic *E. coli* serotype O157:H7 was substantially inhibited by fermentation products from *L. acidophilus* La-5, resulting in the suppression of virulence gene (LEE - locus of enterocyte effacement) expression *in vitro*. This disrupts quorum sensing and eventually prevents GIT colonization by *E. coli* serotype O157:H7 in the GIT (Medellin-Peña et al., 2007).

2.3.5 Immunomodulation

The gastrointestinal tract component of the immune system protecting the host from the different types of antigens in the lumen of the GIT is affected by probiotics. Both innate and adaptive immunity are affected by probiotics.

2.3.5.1 Improvement in innate gut immunity through restitution of intestinal barrier function

Epithelial cells in the gastrointestinal mucosa create a selectively permeable barrier between the intestinal lumen (which contains harmful substances such as foreign antigens, microorganisms, and toxic materials as well as beneficial nutrients) and the internal environment of the body (Blikslager

et al., 2007, Groschwitz and Hogan, 2009). This barrier is the first line of defence against the microbes in the GIT (Baumgart and Dignass, 2002, Peterson and Artis, 2014). It has a combined defence function; incorporating anatomical structures, immunological secretions consisting of mucus, immunoglobulins eg IgA, antimicrobial peptides, and the epithelial junction adhesion complex (Baumgart and Dignass, 2002, Ohland and MacNaughton, 2010). Disease conditions which cause immunological disturbances disrupt this barrier (Turner, 2009), inducing inflammation of the intestinal wall and intestinal disorders (Hooper et al., 2001, Sartor, 2006).

Probiotic formulations prevent chronic inflammation of the GIT through stimulation of innate immunity in the gastrointestinal epithelium (Pagnini et al., 2010, Galdeano and Perdigon, 2006). For example, a high dose (50×10^9 cfu/day) of a probiotic formulation (VSL#3) containing four strains of lactobacilli (*L. casei, L. plantarum, L. acidophilus,* and *L. delbrueckii* subspecies *bulgaricus*); three strains of bifidobacteria (*Bi. longum, Bi. breve, and Bi. infantis*); and one strain of streptococcus (*S. salivarius* subspecies *thermophilus*) when fed to senescence-accelerated-prone mice for six weeks either completely prevented ileitis or significantly reduced the severity of inflammation (Pagnini et al., 2010). Although this probiotic formulation was found to prevent ileitis, it was ineffective in treating the inflammation when administered to older mice that had already developed ileitis (Pagnini et al., 2010).

Experiments in animal models have shown that improvement in intestinal barrier function by probiotics is due to a reduction in the permeability of the intestinal epithelium. Translocation of intestinal microbes out of intestinal sites and into sites such as the liver, spleen and mesenteric lymph nodes decreased in mice with induced colitis and pre-treated with *Lactobacillus* probiotics (Mao et al., 1996, Pavan et al., 2003, Llopis et al., 2005). Translocation of enterotoxigenic *E. coli* to mesenteric lymph node was reduced in post weaning piglets with dietary supplementation of probiotic *P. acidilactici* compared to control group after enterotoxigenic *E. coli* challenge (Lessard et al., 2009).

Generally, timing of probiotic treatment is very important in maintaining intestinal barrier function. Administration of probiotics before the infectious/pathogenic agent is introduced experimentally or the pathogens enter the GIT and multiply naturally is the most effective time for probiotic introduction (Lodemann, 2010).

2.3.5.2 Stimulation or suppression of immune response

The immune response in the host should be sometimes stimulated (for example infection and immunodeficiencies) while it should be supressed in some other cases (for example allergy and

autoimmune diseases) based on the clinical condition (Borchers et al., 2009). Diets containing probiotics could modulate the host immune response.

The responses are complicated as they vary with the probiotic strain or species, with the dose level and may differ in their effect pre and post weaning, and whether the antigen is a bacterium such as Salmonella or a virus such as the human rotavirus.

The pattern of immune response related blood plasma cells can vary between the ileum and jejunum lymph tissue. Probiotics can affect the expression of the anti-inflammatory cytokine or cell signalling proteins and may do so differentially depending on the cytokine. Can probiotics "prime" the immune system in commercial operations to support response to animal and/or human bacterial and viral disease antigens and reduce their shedding in faeces? These are very complicated responses and the variation between probiotic strains means that there is no general "story" about the way probiotics might affect the immune system.

However the significant outcome is that probiotic microbes can modulate the immune system and response to pathogen antigens and a systems based approach is required to address the response to a probiotic in terms of host disease susceptibility, shedding of pathogens both human and/or porcine, growth and feed use efficiency, as a guide to what probiotic a producer may wish to use. It may depend on what is the dominant factor needing to be addressed in the production system. With increasing community (and regulatory) pressure to reduce antibiotic use in commercial animal production, modulation of the immune system by probiotics is a major potential benefit to be factored into production systems.

Several studies have demonstrated immunostimulatory effects of probiotics. Bai et al. (2013) demonstrated that a probiotic containing *L. fermentum* and *S. cerevisiae* stimulated the intestinal T-cell immune system, indicated by increased production of CD3+, CD4+, and CD8+ T-lymphocytes in the GIT of broiler chickens. Expression of CD3+, IL-2 and IFN-γ genes was significantly greater in small intestine of neonatal chicks (day 3 and 7) fed with probiotics *L. jensenii* TL2937 and *L. gasseri* TL2919 than control without probiotics (Sato et al., 2009). Dalloul et al. (2003) found similar effects of probiotics on the intestinal immune system of broiler chickens treated with a commercial probiotic product (Primalac) containing *L. acidophilus, L. casei, E. faecium* and *Bi. Bifidium* and infected with coccidia oocysts, the response being an increased population of intestinal intraepithelial lymphocytes (IEL) cf. control birds not given the probiotic. An increase in expression of CD3+, CD4+, CD8+ and αβTCR (T Cell Receptor - a double chain glycoprotein on the surface of the T cell) was observed. Probiotic *B. cereus* var. toyoi also caused significant increase in the intraepithelial population of CD8+ T cells in intestine of piglets (Scharek et al.,

2007). Similarly, administration of probiotic *E. faecium* to broiler chickens challenged with *E. coli* resulted in increased concentrations of cytokines (IL-4 and TNF- α) and IgA in the small intestinal mucosa (Cao et al., 2013).

Probiotics also increase serum immunoglobulin levels. A multi-strain probiotic containing *L. acidophilus, B. subtilis* and *C. butyricum* increased serum levels of IgA and IgM in chickens (Zhang and Kim, 2014). Likewise, addition of the commercial product (Gallipro) containing *B. subtilis* to broiler chicken diets increased the antibody response to sheep red blood cells administration (Afsharmanesh and Sadaghi, 2014). Antibody titre against the common poultry diseases Newcastle Disease, Infectious Bronchitis and Infectious Bursal Disease was increased by the use of probiotic product Primalac (Landy and Kavyani, 2013).

In the piglets, probiotic *L. fermentum* I5007 modulated immune function in piglets by enhancing T cell differentiation and upregulating ileum cytokine expression (Wang et al., 2009). Similarly, probiotic containing *P. acidilactici* and *S. cerevisiae ssp. boulardii* increased T cells in ileum and IgA secretion in post weaning piglets challenged with enterotoxigenic *E. coli* (Lessard et al., 2009).

In contrast, some studies have shown immunosuppressive action of probiotics in the host. *E. faecium* NCIMB 10415 had an immunosuppressive effect delaying early immune response to antigens in post weaning piglets (Siepert et al., 2014). *E. faecium* NCIMB 10415 reduced proliferation of blood mononuclear cells in response to *Salmonella* serovar *typhimurium* DT104 antigen during 1 to 3 days post-infection followed by a similar proliferative response with or without the probiotic 7 days post-infection (Siepert et al., 2014). Similarly, expression of intestinal immune-associated genes , especially during the post-weaning period, were reduced (Siepert et al., 2014). In the post-weaning period, expression of IL-8, IL-10 and CD86 (cluster of differentiation 86) genes in ileal Peyer's patches was significantly reduced in probiotic treated piglets. In contrast, probiotic caused increased expression of IL-10 gene and CTLA4 (T cell inhibitory molecule) in Jejunal Peyer's patches in post-weaning period. Blood serum inflammation related cytokines IL-6 and IL-8 were not affected by the probiotic.

In an earlier study, supplementation of piglets diet with the same probiotic strain (*E. faecium* NCIMB 10415) had no effect on the lymphocyte populations in the jejunal Peyer's patches (Scharek et al., 2005). The serum level of immunoglobulin IgG was reduced in probiotic treated piglets during the post-weaning period (28-56 days) but was not affected in the pre-weaning period (Scharek et al., 2005).

In another study, oral administration of *L. brevis* ATCC 8287 at the high dose rate of 10^{10} cells per animal per day to weaned piglets reduced expression of IL-4, IL-6 and TGFβ1 genes in the ileum and increased expression of IL-4 and IL-6 gene in the jejunum, caecum and colon (Lähteinen et al., 2014). However this change in cytokine gene expression in the intestine did not change the systemic humoral immune response. Levels of serum immunoglobulins IgA and IgG were the same in control and probiotic treated piglets.

Drenching of *L. acidophilus* strain NCFM at low dose rate (up to 10^6 cfu/dose x 5 doses) significantly increased the population of the antiviral interferon IFN-γ producing T cells and reduced the regulatory T cells and production of TGF-β and IL-10 in intestinal lymphoid tissue of gnotobiotic piglets compared to untreated animals (Wen et al., 2012). In contrast, the same probiotic when administered at a high dose rate (up to 10^9 cfu/dose x 14 doses) increased regulatory T cells.

Such dose dependent response could be one of the reasons for variable results in different studies and with different probiotics. The gastrointestinal microbial profile of the host also could influence the immune response of the host against specific probiotic (Borchers et al., 2009).

2.3.6 Colonization resistance

The GIT of neonatal animals and birds reared naturally are colonized with microorganisms, generally originating from the adult (mother). These microorganisms provide protection from enteric pathogens. Intensification of animal agriculture has reduced the opportunity for natural colonization of the GIT making animals more susceptible to intestinal pathogen challenge. Probiotics could mimic natural colonization in neonates or colonize adult animals preventing pathogenic organisms from colonizing the intestinal mucosa.

Certain strains of *Lactobacillus* and *Bifidobacterium* possess hydrophobic surface layer proteins which help the bacteria to non-specifically adhere to the animal cell surface (Johnson- Henry et al., 2007, Coconnier et al., 1992, Bernet et al., 1994, Hudault et al., 1997, Tuomola and Salminen, 1998, Bibiloni et al., 2001). Such adhesion of probiotic bacteria to the intestinal epithelium covers the receptor binding sites preventing pathogenic microorganisms like *E. coli* O157:H7, *Salmonella* etc., from attaching to the epithelium (Johnson- Henry et al., 2007, Bernet et al., 1994, Hudault et al., 1997).

There are several proposed modes of action of probiotics. Some of these mechanisms are associated with the inhibition of enteric pathogenic microorganisms while others are responsible for improved

animal performance. Different probiotics may have similar mode(s) of action while a specific strain could function through multiple mechanisms. For example, several probiotic strains have similar effects on the gastrointestinal microbial population. However, modes of action of specific probiotics are generally not understood. In most of the studies about effects of probiotics on performance the exact mode of action of probiotics is not fully understood. Because closely related probiotic microorganisms appear to have different modes of action, mechanisms need to be studied on a case-by-case basis. Effects of probiotics are the outcome of interaction between host and probiotic microorganism. Therefore, further studies on host-microbes interaction could elucidate the probiotic mode of action. The rapid advances in molecular methods and DNA sequencing used to study microbial ecology will greatly facilitate our understanding of the way probiotics work.

2.4 Effects of probiotics in poultry

Poultry are the cheapest source of animal protein contributing significantly to supplying the growing demand for animal food products around the world (Farrell, 2013). The consumption and trade in poultry products is increasing rapidly as the human population increases, making it the second largest source of meat after pork (FAO, 2014).

Probiotics are being used in poultry production as an alternative to antibiotic growth promoters. Probiotics can improve broiler chicken growth rates (Afsharmanesh and Sadaghi, 2014, Zhang and Kim, 2014, Mookiah et al., 2014, Lei et al., 2015) and control or prevent enteric diseases, including; salmonellosis (Tellez et al., 2012, Haghighi et al., 2008, Biloni et al., 2013), necrotic enteritis (Jayaraman et al., 2013) and coccidiosis (Dalloul et al., 2003). However the outcomes from probiotic use are not consistent.

2.4.1 Growth rate

Probiotics have enhanced the growth rate in broilers better than AGP (avilamycin) (Zhang and Kim, 2014) and other substitutes for AGP like phytochemicals (e.g. essential oils) (Khaksar et al., 2012). However, the general applicability of the probiotic approach as an alternate for AGP is not yet well established.

Probiotics ranging from non-spore forming LAB to spore formers and yeast have been evaluated for their potential to improve growth rate in commercial poultry production (Afsharmanesh and Sadaghi, 2014, Bai et al., 2013, Shim et al., 2012). In many cases the improvement in growth rate in the probiotic treated birds was associated with increased feed intake (Abdel-Raheem et al., 2012, Landy and Kavyani, 2013, Lei et al., 2015) and improved feed use efficiency (Shim et al., 2012,

Mountzouris et al., 2010, Zhang and Kim, 2014) compared with untreated birds. Therefore, increased digestibility of feed resulting improved feed use efficiency could be one of mode of actions for improved growth rate (also see section 7.1.2 and 7.1.4). Also, the differences in performance between treated and untreated birds may be due to a change in microbial populations in the GIT resulting from increased production of SCFA and immuno-modulation (Zhao et al., 2013). Increased growth rate has also been associated with increased villus height (also see section 7.1.5) which increases absorption of nutrients from the intestine.

In contrast, some probiotics did not improve growth in broilers (Hung et al., 2012, Fajardo et al., 2012, Zhao et al., 2013) even with the same species of probiotic microbe while some probiotics were inferior to AGP in enhancing the growth rate (Abudabos et al., 2015). For example, Cao et al. (2013) found that *E. faecium* (HJEF005) at 10^9 cfu/kg of feed improved growth rate in male Cobb broilers challenged with *E. coli*, while Zhao et al. (2013) using a different strain (LAB 12 – CGMCC 4847), fed at the rate of $2x10^9$ cfu/kg of feed to male Ross broilers, found no growth effect. Use of different broiler breeds in these two studies or different probiotic strains could be the reason for contrasting results. Recent studies suggested that probiotics could be more effective when used with prebiotics (Mookiah et al., 2014). In contrast, Abudabos et al. (Abudabos et al., 2015) reported that body weight gain in the broiler chickens fed the combination of prebiotics and probiotics was less than the birds fed either probiotics or prebiotics individually. "A prebiotic is a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host wellbeing and health" (Gibson et al., 2004).

Effects of probiotics on growth in poultry are detailed in Table 2-1.

One of the interesting observations from probiotic feeding trials in poultry is that some promote growth in the starter (early) phase (Bai et al., 2013) while others affect the grower-finisher (later) phase (Abdel-Rahman et al., 2013, Chawla et al., 2013, Shim et al., 2012) (Table 2). Other studies found improved growth throughout the broiler production cycle (Rahman et al., 2013, Cao et al., 2013, Landy and Kavyani, 2013, Mookiah et al., 2014) (Table 2). The underlying reason for this difference is not known but presumably relates to the dynamics of the gut microbiota. Whether different probiotics should be used in particular growth periods i.e. *choosing the right probiotic for the right time*, remains to be determined.

Many strains of probiotic microbes improve the growth rate of poultry but results can be inconsistent

Table 2-1: Probiotic effects on performance of poultry

Microorganisms (species)	Commercial products*	Growth rate/Body weight gain			Feed intake	FCR			Histomorphology		References
		Prestarter-starter phase	Grower-finisher phase	Over all (lifetime)		Prestarter-starter phase	Grower-finisher phase	Over all (lifetime)	Villus height	Villus height/crypt depth ratio	
B. subtilis	GalliPro, PrimaLac	NS	-	S (+)	S (+)	NS			S (+)	S (+)	(Afsharmanesh and Sadaghi, 2014)
B. subtilis	GalliPro	S (+)	S (+)	S (+)	S (+)	S (-)	S (-)	S (-)	S (+)	-	(Abudabos et al., 2015)
B. subtilis	Super-CyC	NS	S (+)	S (+)	-	NS			-	-	(Abdel-Rahman et al., 2013)
E. faecium	Anta Pro EF	NS	S (+)	S (+)	-	NS			-	-	(Abdel-Rahman et al., 2013)
L. fermentum *S. cerevisiae*	JSA -101 Gold	S (+)	NS	-	S (+)	S (-)	NS	NS	-	-	(Bai et al., 2013)
L. salivarius *P. parvulus*	FloraMax-B11	NS	-	-		-	-	-	S (+)	NS	(Biloni et al., 2013)
L. acidophilus D2/CSL (CECT 4529)	-	S (+)	S (+)	NS	S (-)	NS	NS	S (-)	-	-	(De Cesare et al., 2017)

E. faecium		NS	S (+)								(Chawla et al., 2013)
B. coagulans		NS	NS	NS	NS	S (-)	S (-)	S (-)	NS	NS	(Hung et al., 2012)
B. coagulans		-	-	S(+)	-	-	-	S (-)	-	-	(Zhou et al., 2010)
B. licheniformis *B. subtilis*	Enhancer	NS	S (+)	S (+)	S (+)	S (-)	S (-)	S (-)	-	-	(Abdel-Hafeez et al., 2017)
L. plantarum B1		NS	S (+)	S (+)	NS	NS	S (-)	NS	NS	NS	(Peng et al., 2016)
L. acidophilus, B. subtilis S. cerevisiae A. oryzae		NS	S (+)	S (+)	NS	S (-)	S (-)	S (-)	-	-	(Shim et al., 2012)
L. reuteri *E. faecium* *Bifidobacterium animalis* *Pediococcus acidilactici* *L. salivarius*	PoultryStar ME	NS	S (+)	S (+)	NS	NS	S (-)	S (-)	-	-	(Mountzouris et al., 2010)
C. butyricum		NS	S (+)	S (+)	S (+)	NS	NS	NS	-	-	(Zhao et al., 2013)
E. faecium		NS	NS	NS	NS	NS	NS	NS	-	-	(Zhao et al., 2013)
L. acidophilus, B. subtilis DSM 17299, and C.	Probion	NS	S (+)	S (+)	NS	NS	S (-)	NS	-	-	(Zhang and Kim, 2014)

butyricum.										
L. acidophilus *L. bulgaricus L. plantarum* *S. faecium* *Bi. bifidus* *B. subtilis* *B. licheniformis B. megaterum* *B. mesentricus* *B. polymyxa* *S. bourlrdii*	Microguard	S (+)	S(+)	S(+)	-	-	-	-	-	-	(Rahman et al., 2013)
E. faecium		S (+)	S (+)	S (+)	-	-	-	-	S (+)	S (+)	(Cao et al., 2013)
S. cerevisiae	Bro-bio-fair	-	-	S (+)	S (+)	-	-	S (-)	S (+)	S (+)	(Abdel-Raheem et al., 2012)
L. plantarum L. delbrueckii ssp. bulgaricus *L. acidophilus* *L. rhamnosus* *Bi. bifidum* *S. salivarius ssp. thermophilus* *E. faecium* *A. oryzae* *C. pitolepesii*	Protexin	NS	NS	NS	NS	NS	NS	NS	-	-	(Daskiran et al., 2012)
L. casei subsp. casei CECT 4043		S (-)	-	NS	NS	NS	-	NS	-	-	(Fajardo et al., 2012)

L. lactis subsp. lactis CECT 539		S (-)	-	NS	S (-)	NS	-	NS	-	-	(Fajardo et al., 2012)
L. acidophilus *L. casei* *E. faecium* *Bi. bifidium*	Primalac	S (+)	S (+)	S (+)	S (+)	S (-)	S (-)	S (-)	-	-	(Landy and Kavyani, 2013)
11 Lactobacillus strains (L. reuteri C 1, C 10 and C 16; L. gallinarum I 16 and I 26; L. brevis I 12, I 23, I 25, I 218 and I 211, and L. salivarius I 24)		S (+)	S (+)	S (+)	NS	S (-)	S (-)	S (-)	-	-	(Mookiah et al., 2014)
B. amyloliquefaciens		NS	S (+)	S (+)	S (+)	S (-)	S (-)	S (-)	S (+)	S (+)	(Lei et al., 2015)
B. amyloliquefaciens		S (+)	S (+)	S (+)	S (+)	S (-)	NS	S (-)	-	-	(Ahmed et al., 2014)

S (+) = significantly increased, S (-) = significantly decreased, NS = non-significant, - = not studied, *Details (manufacturer, city and country) of commercial products are given in appendix 1.

2.4.2 Feed intake and feed efficiency

As feed is the largest cost in poultry production, small improvements in feed use efficiency have a significant economic impact. The improvement in performance and productivity of poultry due to the use of probiotics in feed has been attributed to increased feed intake and improved feed efficiency (Shim et al., 2012) but this is not always the outcome. Probiotics can:

- Increase feed intake without significant improvement in feed conversion ratio (FCR) (Afsharmanesh and Sadaghi, 2014)
- Improve FCR without significant difference in feed intake (Mountzouris et al., 2010, Shim et al., 2012, Zhang and Kim, 2014, Zhang et al., 2012) and
- Increase feed intake along with significant improvement in FCR (Landy and Kavyani, 2013).

In contrast, Hung et al. (2012) found that dietary use of the probiotic *B. coagulans* reduced the average daily feed intake by 8% in the broiler grower-finisher phase (day 22-42) with reduction in FCR by 10%. Similarly, Amerah et al. (2013) administered the commercial probiotics (Enviva Pro 202 GT, Danisco Animal Nutrition, Marlborough, UK) containing three strains of *B. subtilis* (strains (BS8, 15AP4 and 2084) during grower/finisher phase of a 42 days feeding trial and found a reduction in feed intake of 2% along with reduction in FCR by 2.7%.. Similarly, Mookiah et al. (2014) found a reduction in feed intake of 5.6% during the starter phase (1-21 days) in birds treated with a multi strain probiotic containing 11 *Lactobacillus* strains (*L. reuteri* C1, C10 and C16; *L. gallinarum* I16 and I26; *L. brevis* I12, I23, I25, I218 and I211, and *L. salivarius* I24). However, FCR was improved in both starter (by 7.3%) and finisher phase (by 12%).

The effect of probiotics on feed intake and feed use efficiency may be growth phase dependent. Some probiotics had no effect on feed intake and FCR during the starter phase while feed intake increased during grower-finisher phase or *vice versa* (Afsharmanesh and Sadaghi, 2014, Chawla et al., 2013, Giannenas et al., 2012, Mookiah et al., 2014).

Many probiotics have positive effects on feed intake and feed use efficiency. However, as with other effects of probiotics, the impact on feed intake and feed use efficiency has not been consistent across studies or with different probiotics.

2.4.3 Carcass yield and quality

Few studies have examined the effects of probiotics on carcass yield and quality in poultry. Marketable carcass yield or ready to cook quantity of carcass at day 42 was increased concurrently with increased growth rate and improved feed use efficiency with the use of the commercial probiotic Anta Pro EF containing *E. faecium* DSM 10663 NCIMB 10415 (in drinking water) and Super-CyC , a mix of the spore forming bacteria *B. subtilis* and a yeast *S. cerevisiae* KCTC 7193 (in feed) (Abdel-Rahman et al., 2013). Anta Pro EF (*E. faecium)* in drinking water at the rate of 2 g per 100 birds per day increased of ready to cook carcass weight and overall body weight gain at day 42 (Abdel-Rahman et al., 2013). In contrast, Afsharmanesh and Sadaghi (2014) did not find any difference in carcass yield, growth rate and feed use efficiency of birds at day 42 treated with a commercial probiotic (GalliPro) containing *B. subtilis.*

Water holding capacity of poultry meat was increased (reduced drip loss) in birds fed with the probiotic *B. coagulans* (Zhou et al., 2010). The tenderness of the meat was also improved in probiotic treated birds in the same study using a local breed of meat type chicken in China. In contrast, Zhang et al. (2005) using another probiotic (*S. cerevisiae*), found no improvement in tenderness in breast meat of commercial broilers. However, both the probiotics had positive effects on growth rate and FCR.

Zhao et al. (2013) found differences in meat quality of Ross broiler chicks between two different probiotics. The intramuscular fat content in breast muscle was increased by 3.6% (1.99 vs 1.92 mg/g) in birds treated with probiotic *C. butyricum* while there was no effect with the probiotic *E. faecium.*

The effect of probiotics on the relationship between carcass quality and yield is unclear – is it due to an effect on muscle or due to improved growth performance *per se*. The inconsistencies in the response may be due to the differences in probiotic strains and/or the breed of birds used.

The effects of probiotics on carcass quality and yield are inconclusive.

2.4.4 Nutrient Digestibility

The apparent ileal digestibility (AID) of essential amino acids was improved in birds fed a maize-soybean based diet supplemented with a low dose (1 to 2 × 10^2 cfu/g) of a multi strain commercial probiotic (Probion) containing *L. acidophilus, B. subtilis* and *C. butyricum* (Zhang and Kim, 2014). All essential amino acids, except histidine and phenylalanine, had improved AID in treated birds as

compared to control birds, but there was no effect of probiotics on digestibility of DM, nitrogen and energy. However, Li et al. (2008), found an increase in the apparent digestibility of DM, energy, CP, Ca, P and amino acids in male broilers fed corn-soybean based diet supplemented with commercial probiotic (AgiPro A100) containing yeast and other microbes. Interestingly, digestibility of nutrients in grower-finisher phase was higher than in the starter phase. Apata (2008) also found that the probiotic *L. bulgaricus* could improve apparent ileal digestibility of DM and CP in broiler chicken fed maize-soybean based diet. Similarly, Chawla et al. (2013) found the probiotic *E. faecium* increased blood calcium levels in Vencobb broiler chicks indicating improved bioavailability. Different strains of probiotic microbes produce different enzymes and understanding the effects these might have on different feed ingredients would help understanding of the way probiotics might "work" for animal production.

Probiotics can improve nutrient digestibility in poultry, but the interaction with different feedstuffs used in poultry diets is little understood at present.

2.4.5 Intestinal Histomorphology

The structure of the intestinal mucosa is an important determinant of intestinal function (digestive and absorptive) affecting growth performance of poultry. Generally, increases in villus height and villus height:crypt ratio increases the absorption of nutrients due to a larger surface area (Afsharmanesh and Sadaghi, 2014).

Probiotics in poultry diets can affect the histology of the intestinal mucosa. The villus height and the villus:crypt ratio in the intestinal mucosa were increased by *B. subtilis* (Afsharmanesh and Sadaghi, 2014, Jayaraman et al., 2013), *B. coagulans* (Hung et al., 2012), the lactic acid producing bacteria *L. salivarius*, *P. parvulus* (Biloni et al., 2013) and *E. faecium* (Cao et al., 2013, Abdel-Rahman et al., 2013).

Villus height in probiotic (*B. coagulans* ATCC 7050) treated birds was greater than in birds treated with an AGP (zinc–bacitracin) when measured at 6 weeks age (Hung et al., 2012). Similarly, the probiotic *B. subtilis* PB6 reconstituted the normal structure of chicken intestinal villi distorted and damaged by necrotic enteritis caused by *Cl. perfringens* (Jayaraman et al., 2013).

Some probiotics affect intestinal histomorphology favourably.

2.4.6 Control or prevention of enteric pathogens

The public health risk from zoonotic pathogens of poultry like salmonella and campylobacter and antibiotic resistance is increasing with intensification of the poultry industry in developing countries and imprudent use of antibiotics in animal production systems (Singer et al., 2003, van den Bogaard and Stobberingh, 2000). In addition, other enteric diseases of poultry, like necrotic enteritis and coccidiosis, cause huge economic losses to the industry (Bera et al., 2010, Williams, 1999, Skinner et al., 2010). The change in the poultry production systems which result in delayed colonization of the gastrointestinal mucosa by healthy microflora may be one of the reasons for the increasing incidence of enteric pathogens (Crhanova et al., 2011). The virtually sterile environment immediately post-hatch, makes it possible for opportunistic pathogens to colonize the intestine (Flint and Garner, 2009). Probiotics may prevent or control such enteric pathogens.

2.4.6.1 Salmonellosis

Salmonellosis in poultry is a significant food safety issue as the pathogen causes a major food borne illness in humans. Successful use of undefined gastrointestinal culture for the prevention and control of *Salmonella* infection in chicken by Nurmi and Rantala (1973) led to many studies about use of gastrointestinal culture and probiotics to control salmonella in poultry (Lloyd et al., 1977, Snoeyenbos et al., 1979, Bolder et al., 1992). Competitive exclusion between pathogenic and non-pathogenic ingested bacteria was believed to be the mechanism preventing infection in earlier studies.

Probiotics are emerging as an alternative salmonella control method which also addresses the increasing concern about antibiotic resistant strains of *Salmonella* (Tellez et al., 2012). Haghighi et al. (2008) demonstrated that probiotics could reduce the caecal colonization by *Salmonella* by several fold (1.2 to 3.0 $\log_{10}$) depending on probiotic dose. With a single application at dose rate of 1×10^5 and 1×10^6 cfu of a commercial probiotic product containing *L. acidophilus, Bi. bifidum,* and *S. faecalis*, the larger dose rate caused a larger reduction in the caecal *Salmonella* population.

The protection against *Salmonella* colonization appeared linked to a change in cytokine expression (IFN-γ and IL-12) in gut-associated lymphoid tissue. Some probiotics produce SCFA in the caeca in sufficient amounts to inhibit *Salmonella enterica* serovar Enteritidis (Argañaraz-Martínez et al., 2013). By using an *in vitro* test, Argañaraz-Martínez et al. (2013) demonstrated that SCFA production in the caeca of chickens treated with *Propionibacterium acidipropionici* LET 105 was 30% greater than in the control birds. This probiotic also competed with *Salmonella* for adhesion to the intestinal mucosa (Argañaraz-Martínez et al., 2013). Probiotics also reduced the spread of

Salmonella from infected to healthy birds. Transmission of *Salmonella* infection within the flock (horizontal transmission) was slower with a probiotic containing *L. salivarius* and *Pediococcus parvulus* (Biloni et al., 2013).

2.4.6.2 Campylobacteriosis

Campylobacteriosis is an important zoonotic disease of poultry caused by *Ca. jejuni*. *In vitro* experiments with probiotic bacterial strains *(E. faecium, P. acidilactici, L. salivarius,* and *L. reuteri)* isolated from the GIT of healthy chickens showed that they could inhibit growth of *Ca. jejuni* on agar plates (Ghareeb et al., 2012). The result was confirmed *in vivo* with broiler chickens. Inhibition of growth *in vitro* suggests production of growth inhibiting compound by probiotics. Combination of four bacterial strains *Lactobacillus paracasei J.R, L. rhamnosus 15b, L. lactis Y, and L. lactis FOa* prevented the invasion and colonisation of *Ca. jejuni* in the duodenum and cecum (Cean et al., 2015). Similarly, the commercial probiotic Primalac (containing *Lactobacillus*, *Bifidobacterium* and *Enterococcus*) reduced the prevalence of *Campylobacter* infection in broiler chickens (Willis and Reid, 2008). Morishita et al. (1997) had earlier demonstrated that oral administration (via drinking water) of a commercial probiotic containing a mixture of *L. acidophilus* and *S. faecium*, to broiler chickens, during the first 3 days of life, reduced the shedding of *Campylobacter* by 70% in artificially infected birds and decreased the intestinal colonization by *Campylobacter* by 27%.

2.4.6.3 Necrotic enteritis

Necrotic enteritis (NE) caused by *Cl. perfringens* is an economically important disease in poultry due to the high prevalence of losses (Hermans and Morgan, 2007, McDevitt et al., 2006) causing significant economic loss to the industry worldwide (Van der Sluis, 2000, Timbermont et al., 2011).

Probiotics are being studied as an alternative to antibiotics to prevent necrotic enteritis in poultry (Caly et al., 2015). Administration of *B. subtilis* (strain PB6) to broiler chickens artificially infected with *Cl. perfringens* reduced the severity of intestinal lesions and significantly reduced the number of pathogen cells in the GIT (Jayaraman et al., 2013, Abudabos et al., 2015). *B. subtilis* strain PB6 produces a heat resistant and anticlostridial factor which could be used to control clostridial infections caused by *Cl. perfringens* and *Cl. difficile* (Teo and Tan, 2005). Negative effect of subclinical *Cl. perfringens* infection on lipid content and fatty acid composition in chicken meat was ameliorated by *Lactobacillus johnsonii* administration in diet (Wang et al., 2017).

2.4.6.4 Coccidiosis

Coccidiosis is the most important protozoan parasitic disease of poultry due to its ubiquitous nature, high rate of resistance to anticoccidial drugs and severe economic consequences for infected flocks (Williams, 1999). The disease is caused by different species of *Eimeria* protozoa that colonize different sections of the GIT. Studies evaluating the effects of probiotics on coccidiosis gave inconclusive results (Lee et al., 2007, Dalloul et al., 2003). However, Giannenas et al. (2012) found a reduction in coccidiosis by using probiotics based on *E. faecium, B. animalis, L. reuteri* and *B. subtilis*, either singly or in combination.

The probiotics were thought to maintain intestinal health in infected birds and significantly reduce the shedding of oocysts from infected birds thereby reducing the spread of disease (Giannenas et al., 2012, Dalloul et al., 2003, Abu-Akkada and Awad, 2015).

Probiotics could be a potential alternative to antibiotic feed additives to manage the enteric pathogen load in poultry, by reducing intestinal colonization and spread of common zoonotic and other enteric pathogens.

2.4.7 Prevention of bacterial chondronecrosis with osteomyelitis

Bacterial chondronecrosis with osteomyelitis (BCO) is a major cause of lameness in chicken predominantly caused by *Staphylococcus aureus* infection of the proximal epiphyseal plate of the femur, tibiotarsus and flexible thoracic vertebrae (McNamee and Smyth, 2000, Wideman Jr et al., 2013, Wideman Jr, 2015, Jiang et al., 2015, Mandal et al., 2016). BCO is suggested to be caused by translocation of pathogenic bacteria through impaired tight junction from the intestine to the predilection site via the systemic circulation (Wideman Jr and Prisby, 2013, Wideman Jr, 2016). The incidence of lameness caused by wire-flooring induced BCO was reduced by at least 50% in chickens fed the probiotics containing *Enterococcus faecium, Bifidobacterium animalis, Pediococcus acidilactici, Lactobacillus reuteri, Enterococcus faecium* and *Bacillus subtilis* (Wideman Jr, 2016, Wideman Jr et al., 2012, Wideman Jr et al., 2015). The suggested mechanism to reduce the BCO lameness in chickens by probiotics are inhibition of pathogenic microbes in the gut, enhanced integrity of gut wall to prevent the translocation of microbes and stimulation of immune system to eliminate the microbes translocated into systemic circulation (Wideman Jr, 2016).

2.4.8 Egg production and quality

While probiotics can affect the production, feed use efficiency and quality of eggs in laying hens, these effects have been very inconsistent (Table 3). Studies showing increase in egg production with supplementation of diets with probiotics (Gallazzi et al., 2009, Kurtoglu et al., 2004, Yörük et al., 2004, Xu et al., 2006), contrast with those showing no effect on egg production (Mikulski et al., 2012, Salma et al., 2007, Asli et al., 2007, Capcarova et al., 2010, Dizaji and Pirmohammadi, 2009). Similarly, there are variable effects of probiotics on feed use efficiency in laying hens. One of the most promising effects of probiotics on egg quality is the consistent reduction of cholesterol in egg yolk. Yolk cholesterol has been reduced by lactic acid bacteria (Haddadin et al., 1996, Panda et al., 2003), *Bacillus* spores (Kurtoglu et al., 2004) and yeast (Yousefi and Karkoodi, 2007).

Table 2-2: Probiotic effects on egg production and quality

Microorganisms	Egg production	FCR (feed weight/egg weight)	Quality of egg					References
			Weight	Egg shell thickness	Yolk cholesterol	Albumin viscosity (Haugh unit)	Specific gravity	
L. acidophilus D2/CSL	S (+)	S (-)	NS	NS	-	S (+)	S (+)	(Gallazzi et al., 2009)
P. acidilactici	NS	S (-)	S (+)	-	S (-)12%	-	S (+)	(Mikulski et al., 2012)
R. capsulatus	NS	NS	-	NS	S (-)26%	NS	-	(Salma et al., 2007)
L. plantarum, L. bulgaricus, L. acidophilus, L. rhamnosus, B. bifidum, S. hermophilus, E. faecium, A. oryzae and C. pintolopessi	NS	-	-	NS	NS	NS	-	(Asli et al., 2007)
S. cerevisiae	NS	-	-	NS	NS	NS	-	(Asli et al., 2007)
B. licheniformis and B. subtilis	S (+)	S (-)	NS	-	S (-)38%	-	NS	(Kurtoglu et al., 2004)

Lactobacillus spp., Bifidobacterium spp., Streptococcus spp., and Enterococcus spp.	S (+)	S (-)	NS	-	-	-	-	(Yörük et al., 2004)
L. acidophilus, L. casei, Bi. bifidum, A. oryzae, S. faceium and Torulopsis spp.,	S (+)	NS	NS	S (+)	S (-)	-	-	(Panda et al., 2003)
E. faecium	NS	-	NS	-	-	-	-	(Capcarova et al., 2010)
S. cerevisiae (strain NCYC sc 47)	NS	S (+)	S (-)	-	-	-	-	(Dizaji and Pirmohammadi, 2009)
B. subtilis (CH201) and *B. lichenioformis* (CH200)	NS	S (+)	S (-)	-	-	-	-	(Dizaji and Pirmohammadi, 2009)
L. acidophilus	S (+)	S (+)	-	NS	S (-)	-	-	(Haddadin et al., 1996)
S. cerevisiae	NS	NS	NS	S (+)	-	-	-	(Hassanein and Soliman, 2010)
E. faecium	NS	S (-)	NS	NS	NS	S (-)	-	(Hayirli et al., 2005)
B. subtilis and *B. lichenioformis*	NS	NS	NS	NS	S (-)	NS	-	(Mahdavi et al., 2005)
B. subtilis	S (+)	S (-)	NS	-	-	-	-	(Xu et al., 2006)

S. cerevisiae	NS	NS	NS	NS	S (-)	-	-	(Yousefi and Karkoodi, 2007)
L. plantarum, *L. delbrueckii ssp. bulgaricus* *L. acidophilus* *L. rhamnosus* *Bi. bifidum* *S. salivarius ssp. thermophilus* *E. faecium* *A. oryzae* *C. pitolepesii*	NS	S (-)	NS	-	-	-	NS	(Balevi et al., 2001)

S (+) = significantly increased, S (-) = significantly decreased, NS = non-significant, - = not studied

2.5 Microbiology of chicken GIT

The gastrointestinal tract of the chicken is an intricate ecosystem which harbors a complex and dynamic consortium of microorganisms, both in the lumen and on the mucosal surface, consisting of more than 640 species of bacteria, bacteriophage and other viruses together with large numbers of protozoa, fungi and methanogenic archaea, which are co-existing with mutual benefit to each other and to the host under the normal circumstances (Yeoman et al., 2012, Saengkerdsub et al., 2007a, Saengkerdsub et al., 2007b, Apajalahti et al., 2004).

Due to apparent effects of gastrointestinal microbial dynamics in animal production systems, on food safety and environment, significant effort has been made to understand the characteristics and function of the chicken gastrointestinal ecology for more than four decades. Earlier studies mostly relied on culture based techniques (Salanitro et al., 1974) and had seriously underrepresented the richness of this ecosystem due to methodological limitations. Traditional culture dependent techniques are unable to cultivate and study the majority of the microorganisms present in the GIT and thus unable to unravel the complexity of this ecosystem to understand the composition, diversity, role and interaction of its members. However, recent developments in culture independent techniques through the use of molecular biology tools and bioinformatics have enabled an in-depth study of this ecosystem with an increasing database and understanding (van der Hoeven-Hangoor et al., 2013, Thompson et al., 2008, Torok et al., 2009, Cressman et al., 2010, Pissavin et al., 2012, Ammor et al., 2008). Particularly, the use of the 16S rRNA gene as a phylogenetic marker to study the microbial diversity, and the development of high-throughput DNA sequencing technologies have enabled the generation of this knowledge at unprecedented speed. There is an increasing thrust to modify the gastrointestinal ecology for the benefit of the host, producer, consumer or the environment by using different types of feed additives. Probiotics are one such feed additive used to modify composition and function of intestinal microbes for improved health or production of the animal.

However, It has been postulated that it is less important to know which bacterial species are present than to know the overall function of the microbiome (Danzeisen et al., 2011).

Bacteria form the most diverse microbial population (Yeoman et al., 2012) and probably, functionally, are the most important sector of the GIT environment. However, there are more bacteriophages than bacteria in the GIT (Rodriguez-Valera et al., 2009). Moreover, there is considerable variation in the microbial community present in the lumen of different sections of the gastrointestinal tract and the corresponding mucosa (Gong et al., 2007, Apajalahti et al., 2004, Apajalahti et al., 2001, Saengkerdsub et al., 2007a) (Figure 2-1). The population and composition of

this ecosystem are contingent mainly on the host diet (Apajalahti et al., 2001) and to some extent on the systemic response and local secretions in the host and on the type of litter material used (Torok et al., 2009). Composition, physical state and nutrient concentration of the animal diet and presence of feed additives have significant effects on the intestinal microbial dynamics (Thompson et al., 2008, Knarreborg et al., 2002b, Singh et al., 2013, Engberg et al., 2000, Engberg et al., 2004). This microbial dynamic has profound effects on health, performance and physiological state of the host animal due to host-microbe interaction influencing nutrient digestion, absorption and immunity (Round and Mazmanian, 2009, Nicholson et al., 2005).

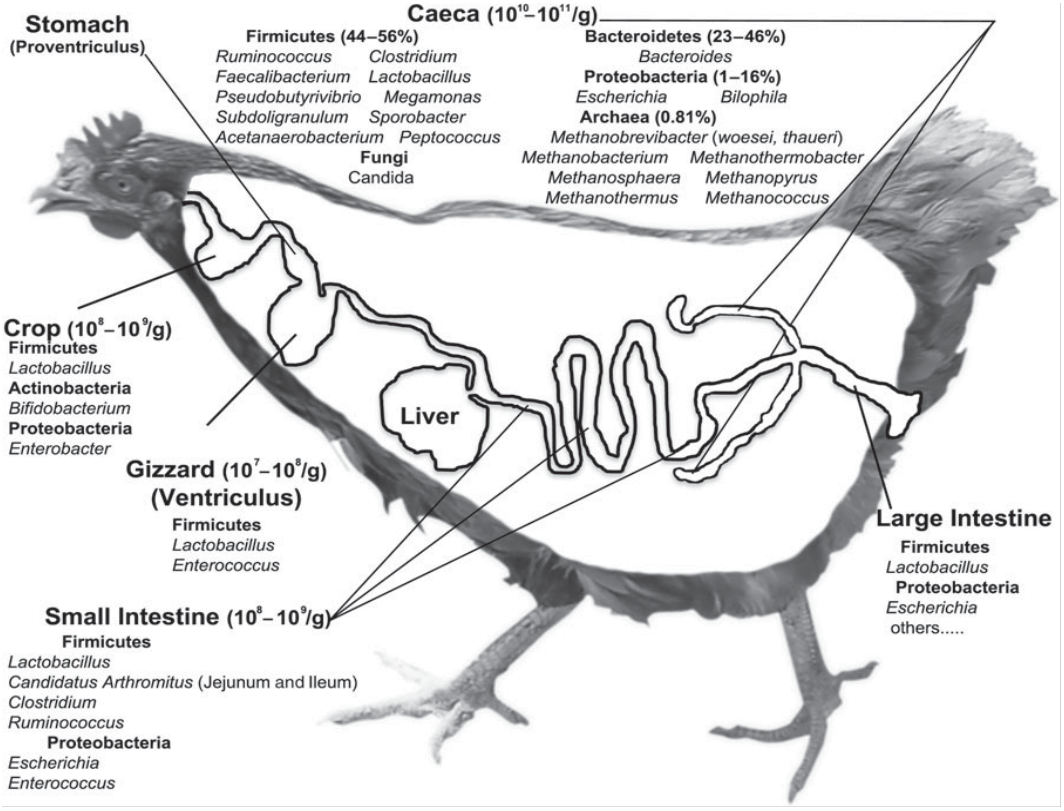


Figure 2-1: Major microbial taxa reported from different sections of the gastrointestinal tract of chicken. Picture reproduced from Yeoman et al. (2012). Data in the figure are taken from Ammor et al. (2008), Saengkerdsub et al. (2007a), Saengkerdsub et al. (2007b) and Gong et al. (2002).

Most of the studies about the microbiome of the chicken GIT have been done in broiler chickens. Therefore, the majority of the available information represents the microbiome of younger chickens. Dominant bacteria in 7-day old chickens are in the family Clostridiaceae both in the ileum and the caecum (Shaufi et al., 2015, Corrigan et al., 2015) while that in 35-day old chickens are in the family Lactobacillaceae in the ileum and family Lachnospiraceae in the Caecum (Pourabedin et al., 2015) (Figure 2-2). The richness and diversity of microbial populations is greater in older birds than those in younger chickens, and the microbial population in the caeca shifts from simple and transient to complex and stable with age (Danzeisen et al., 2011, Videnska et al., 2014).

Chicken crop is generally dominated by *Lactobacillus* sp. (Stanley et al., 2014, Rehman et al., 2007a). Previous studies have revealed that Lactobacillales (phylum Firmicutes), predominantly *Lactobacillus* and *Enterococcus*, and various species under Clostridiaceae were the dominant bacteria from the crop to the ileum, representing more than one third of total sequence reads (Choi et al., 2014, Kohl, 2012, Pan and Yu, 2014, Waite and Taylor, 2014, Stanley et al., 2014). Bjerrum et al. (2006) isolated 92 strains of anaerobic bacteria from the ileum of organically grown broilers and identified them by 16S rRNA gene sequencing. The lactic acid producing bacteria *Lactobacillus reuteri* and *Lactobacillus salivarius* were the two dominant isolates among these. In contrast, anaerobic bacteria such as *Alistipes*, unclassified Ruminococcaceae and unclassified Lachnospiraceae were the most prevalent bacteria from the large intestine (Choi et al., 2014).

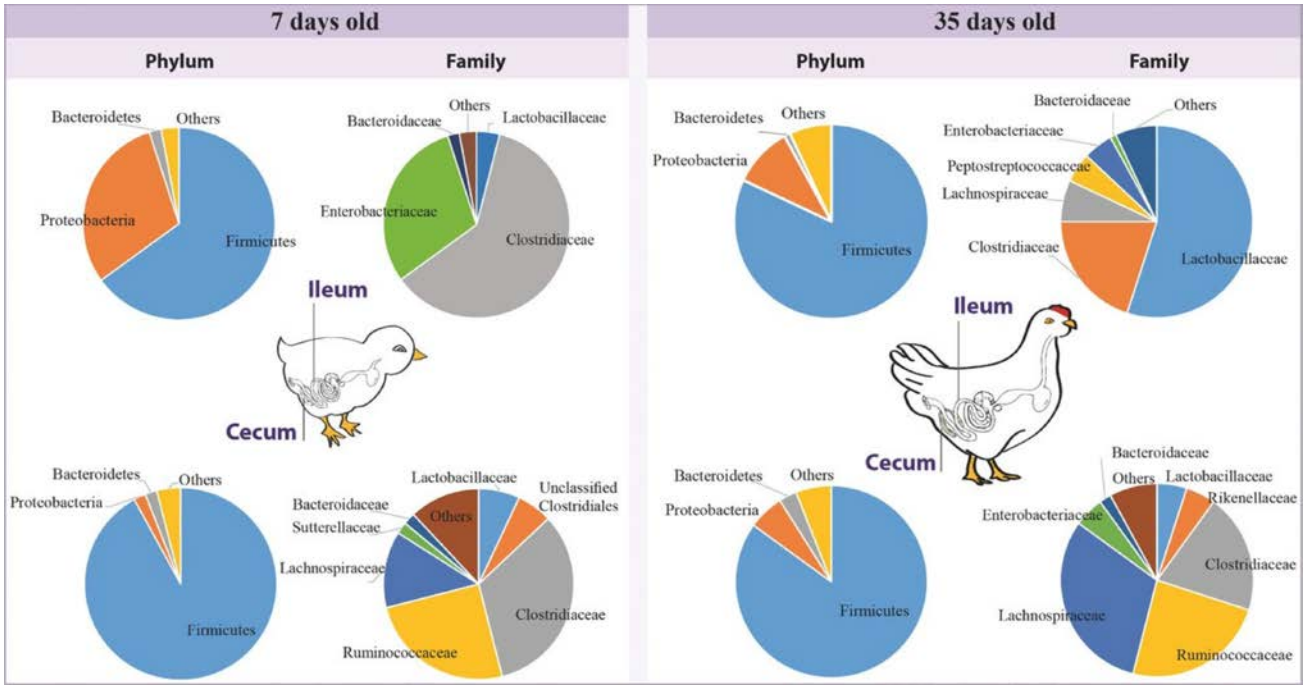


Figure 2-2 Dominant microbiome (phylum and family) in the ileum and the caecum of the chickens at day 7 and day 35. Figure reproduced from Pourabedin and Zhao (2015). Data from Shaufi et al. (2015), Corrigan et al. (2015) and Pourabedin et al. (2015).

The caeca contain the most abundant and the most diversified microbial community of the GIT (Gong et al., 2007), containing about 10^{10} – 10^{11} bacteria per gram fresh weight of caecal digesta (Yeoman et al., 2012). Qu et al. (Qu et al., 2008) reported comparable number of 16s rRNA gene sequences resulted from pyrosequencing studies on the chicken caecum and the bovine rumen. Firmicutes are the most abundant taxa representing more than half of the bacterial population (Qu et al., 2008, Lu et al., 2003, Danzeisen et al., 2011) while 23-46% are Bacteroidetes and 1-16% are Proteobacteria (Yeoman et al., 2012). Archaea represent 0.8% of the caecal microflora (Qu et al., 2008). However, recent studies showed that microbial profile of the chicken GIT can vary considerably (Stanley et al., 2013).

Recent microbial profiling studies using 16S rRNA gene sequences reported as many as 3500 genotypes (Qu et al., 2008) and more than 2300 operational taxonomic units (OTU's) at 95% DNA sequence similarity (Danzeisen et al., 2011). Due to this microbial diversity, high number of microbes and the longer digesta transit time in caeca, microbial fermentation is the most active in this region (Rehman et al., 2007b).

Choi et al. (2014) and Lu et. al. (2003) found Clostridia to be the dominant bacteria in the chicken caecum. Metagenomic sequencing by Danzeisen et al. (2011) showed that OTUs classified as *Roseburia*, a butyrate producing organism from the Lachnospiraceae family, were the most abundant OTUs in the caeca representing about 19% of total sequence reads. A spore forming gram positive anaerobic bacterium, *Sedimentibacter*, was also present (Danzeisen et al., 2011). In contrast, *Ruminococcus* was earlier reported as the dominant bacteria in the caeca followed by *Streptococcus, Bacteroides, Clostridium, Fusobacterium* and *Bifidobacterium* (Apajalahti et al., 2001).

Limited gene-based microbial studies on the function and gene expression of the microbial community in the chicken GIT have been done so far, and those that have been were mainly on the caecal microbial community (Qu et al., 2008, Danzeisen et al., 2011). The metagenome of the chicken GIT contains about 200,000 genes (Qu et al., 2008). The most prevalent functional groups in the metagenome were related to carbohydrate utilization, protein metabolism, and amino acid synthesis (Danzeisen et al., 2011). Firmicutes were responsible for encoding more than 95% of the genetic information in the caeca while Archaea encoded only 1-2% of the genetic information (Qu et al., 2008, Danzeisen et al., 2011).

In the first extensive culture based study of the microbial community of the chicken GIT, Salanitro et al. (Salanitro et al., 1974) isolated 325 strains of culturable bacteria from the caeca of 5-week old hens. These strains have been reported to cover more than 80% of culturable microbes in the caeca. Videnska et al. (2014) studied the microbial ecology of layer chickens over the production lifespan (0 to 60 weeks) using pyrosequencing of V3/V4 regions of 16S rRNA genes and examined the caecal microbiota in four different phases or time periods. Phase one was the first week post-hatch with Enterobacteriaceae as dominant bacterial family. During the second phase from week two to week four, Lachnospiraceae and Ruminococcaceae were the dominant bacterial families. In the third phase from eight to 24 weeks was characterised by increased Firmicutes and decreased Bacteroidetes populations. In the final or fourth phase (egg production phase) from 28 weeks was characterised by a constant ratio of Firmicutes to Bacteroidetes.

2.6 Bacillus amyloliquefaciens

2.6.1 General introduction and taxonomy

B. amyloliquefaciens is a gram-positive endospore forming aerobic bacterium which was initially classified *B. subtilis* subsp. amyloliquefuciens (Tsuru, 1962) and approved as a separate species in 1987 (Priest et al., 1987). It is phenotypically very similar to *B. subtilis* and an important species used in the fermentation industry due to its ability to produce various secondary metabolites (Table 2-3). This bacterium produces extracellular enzymes, e.g. α-amylase, cellulase, proteases and metalloproteases (Gould et al., 1975, Lee et al., 2008, Gangadharan et al., 2008) which are believed to be beneficial in digestion in poultry (Gracia et al., 2003). Several potent antimicrobial compounds belonging to lipopeptide and polyketide groups (table 3) are presumed to be antipathogenic in plants and animals (Chen et al., 2009, Kadaikunnan et al., 2015). *B. amyloliquefaciens* also produces bacteriocins with bactericidal properties against different bacteria including foodborne *Salmonella sp*. *Listeria monocytogenes* and *Shigella sp* (Kaewklom et al., 2013).

This bacterium is a common soil and phyllosphere organism and has also been used in agriculture as plant growth promoter and biocontrol agent against plant pathogens (Chen et al., 2009, Koumoutsi et al., 2004, Liu et al., 2010). The growth promoting effects are attributed to the secondary metabolites produced (Chen et al., 2007).

Table 2-3: Secondary metabolites produced by *B. amyloliquefaciens*

Group	Metabolite	Major characteristics	
Lipopeptides	Surfactin	• Cyclic lipopeptide • Amphiphilic • Characteristic "horse saddle" conformation • Antibacterial, antiviral, antifungal, antiprotozoal, anti-mycoplasma and hemolytic • Non specific cytotoxicity • Can alter membrane integrity	(Hue et al., 2001, Koumoutsi et al., 2004, Sun et al., 2006)
	fengycin	• Antifungal • Inhibits phospholipase A2	(Ongena and Jacques, 2008, Nishikiori et al., 1986, Sun et al., 2006)
	bacillomycin D	• Antifungal • Hemolytic	(Chen et al., 2009, Koumoutsi et al., 2004)
	iturin A	• Antifungal • Hemolytic	(Ongena and Jacques, 2008, Thimon et al., 1995, Arrebola et al., 2010)
polyketides	Macrolactin		(Schneider et al., 2007, Chen et al., 2009)
	Difficidin	Antibacterial	(Chen et al., 2006, Chen et al., 2009, Zimmerman et al., 1987)
	Bacillaene		(Chen et al., 2006, Chen et al., 2009)
	Chlorotetaine		(Rapp et al., 1988)
iron-siderophore	bacillibactin		
Enzymes	α-amylase	Hydrolysis of starch	(Gangadharan et al., 2008, Gracia et al., 2003)
	cellulase	Improve the digestion of nutrients	(Lee et al., 2008)
	proteases	Improve the digestion of nutrients	(Gould et al., 1975)
Bacteriocin	barnase	Antibacterial	(Ulyanova et al., 2011)

2.6.2 Bacillus amyloliquefaciens as probiotic in poultry

B. amyloliquefaciens has been tested as a probiotic in poultry (Lei et al., 2014, Lei et al., 2015) and in fish (Das et al., 2013, Huang et al., 2015) to improve feed conversion efficiency and growth.

Recently, a probiotic product containing three strains of *B. amyloliquefaciens* spores when fed to male broiler chickens on a corn-soya based diet resulted in increased body weight gain, increased feed intake and improved feed conversion ratio (FCR) (Lei et al., 2015). Similarly, the digestibility of the feed during starter phase has also been significantly improved as indicated by higher apparent total tract digestibility of CP, DM and Gross Energy (GE). The improvement in performance of the birds in this experiment was presumed to be attributed to changes in histomorphology of the gastrointestinal tract as demonstrated by increased villus height, reduced crypt depth and increased villus height to crypt depth ratio in the duodenum, jejunum, and ileum; improved nutrient utilization and modified caecal microflora population (decreased *E. coli* and increased *Lactobacillus* populations). Similar effects of *B. amyloliquefaciens* occurred with broiler performance with a significant impact on average body weight gain, feed intake and FCR (Ahmed et al., 2014). Moreover, concentrations of serum IgG and IgA in birds fed *B. amyloliquefaciens* were significantly increased. Lei et al. (Lei et al., 2014) found that *B. amyloliquefaciens* could replace antibiotic growth promoters in the second half of the broiler life cycle, improving productivity of the birds by enhancing nutrient digestibility.

In a previous study, broilers fed a commercial probiotic (Ecobiol) containing *B. amyloliquefaciens* at the dose rate of 10^6 cfu/g of feed attained marketable weight 2.5 days earlier than the birds fed the control diet (Ortiz et al., 2013). Similarly the same commercial product improved the feed conversion ratio in broilers (Diaz, 2007).

Modern intensive poultry production systems have often been criticized for their negative impact on the environment due to emission of ammonia (NH_3) and hydrogen sulfide (H_2S). Microbial urease produced by microorganisms in the gastrointestinal tract of poultry hydrolyze the uric acid produced in the liver and excreted into the gastrointestinal tract to produce ammonia (Karasawa et al., 1988). Similarly, there are several bacterial species and genera in the gastrointestinal tract of poultry which reduce sulfate to produce hydrogen sulfide (Barbour et al., 1985). Ahmed et. al (Ahmed et al., 2014) demonstrated that *B. amyloliquefaciens* can reduce the environmental impact of poultry production by significantly reducing the amount of ammonia (NH_3) and hydrogen sulfide (H_2S) emission from poultry manure. Better nutrient utilization and modification in gastrointestinal

microbial populations were postulated to be the causes for reduced noxious gas emission in this study (Ahmed et al., 2014).

2.6.3 Safety of Bacillus amyloliquefaciens

B. amyloliquefaciens has not been found associated with any infections or toxicity in humans or animals. European Food Safety Authority (EFSA) has given the *B. amyloliquefaciens* Qualified Presumption of Safety (QPS) status (European Food Safety Authority, 2008). EFSA has been using the QPS concept as a generic risk assessment tool to assess the safety of microorganisms intended for deliberate entry into the food chain. Identification of the *B. amyloliquefaciens* with QPS status means this microorganism either does not pose any safety risk or risk could be clearly defined and eliminated (European Food Safety Authority, 2007). Therefore, this microorganism if intended to enter the market in EU may not be subjected to detail pre-market safety assessment other than satisfying predetermined specific qualifications (European Food Safety Authority, 2007). This microorganism is not in the list of Generally Recognized As Safe (GRAS) category of the United States Food and Drug Administration (USFDA) (USFDA, 2013). However, bacterially-derived carbohydrase and protease enzyme preparations from a non-toxigenic and non-pathogenic strain of *B. amyloliquefaciens* are listed as GRAS (US Government Publishing Office, 2013).

2.7 Methods to study the gastrointestinal microbiome

2.7.1 Microbial community study by genomic approaches

Every microbial community has its specific structure, function and ecosystem (Weinstock, 2012). Discovery of culture independent genomic methods has made the study of these communities possible. The structure and functions of microorganisms in the past (before 1980s) were generally studied on the basis of phenotypic characteristics, growth on a range of media (if they were culturable), enzymatic activity, staining characteristics and metabolism of cultured microorganisms (Clarridge, 2004, Petti et al., 2005).

The 16S rRNA gene, which encodes small subunit ribosomal RNA, is one of the most widely used molecular markers in the study of microbial community structure. There are some unique characteristics of this gene, which has made it an "ultimate molecular chronometer" (Woese, 1987). All bacterial species carry at least one copy of this gene and it has the same function in all microorganisms (Woese, 1987). In addition, the 16S rRNA gene consists of highly conserved and variable nucleotide sequences (Olsen et al., 1986, Case et al., 2007). The conserved sequences remain stable across all microorganisms which make the comparison between distantly related microorganisms possible while the variable regions are unique to each group (species/strains) of microorganism which helps to assess the similarity between closely related organisms (Olsen et al., 1986, Case et al., 2007). In addition, large mutations and horizontal gene transfer in the 16S rRNA gene which might disrupt gene function are very rare, as cells cannot survive without ribosomes. Universal primers, which hybridise to highly conserved sequences, bind to all 16S rRNA genes present in the DNA sample and amplify all of these genes during the PCR reaction. The amplified DNA can then be sequenced using next generation sequencing technologies. The DNA sequences are then either compared with the sequences present in web based database applications like Greengenes (DeSantis et al., 2006) and the ribosomal database project (Cole et al., 2005, Cole et al., 2009) to create a table of taxa and their abundances or the sequences could be assembled into operational taxonomic units (OTUs) to form an OTU table and their abundances (Weinstock, 2012) (Figure 2-3). The OTUs which have at least 97% DNA sequence similarity are generally regarded as a species (Weinstock, 2012).

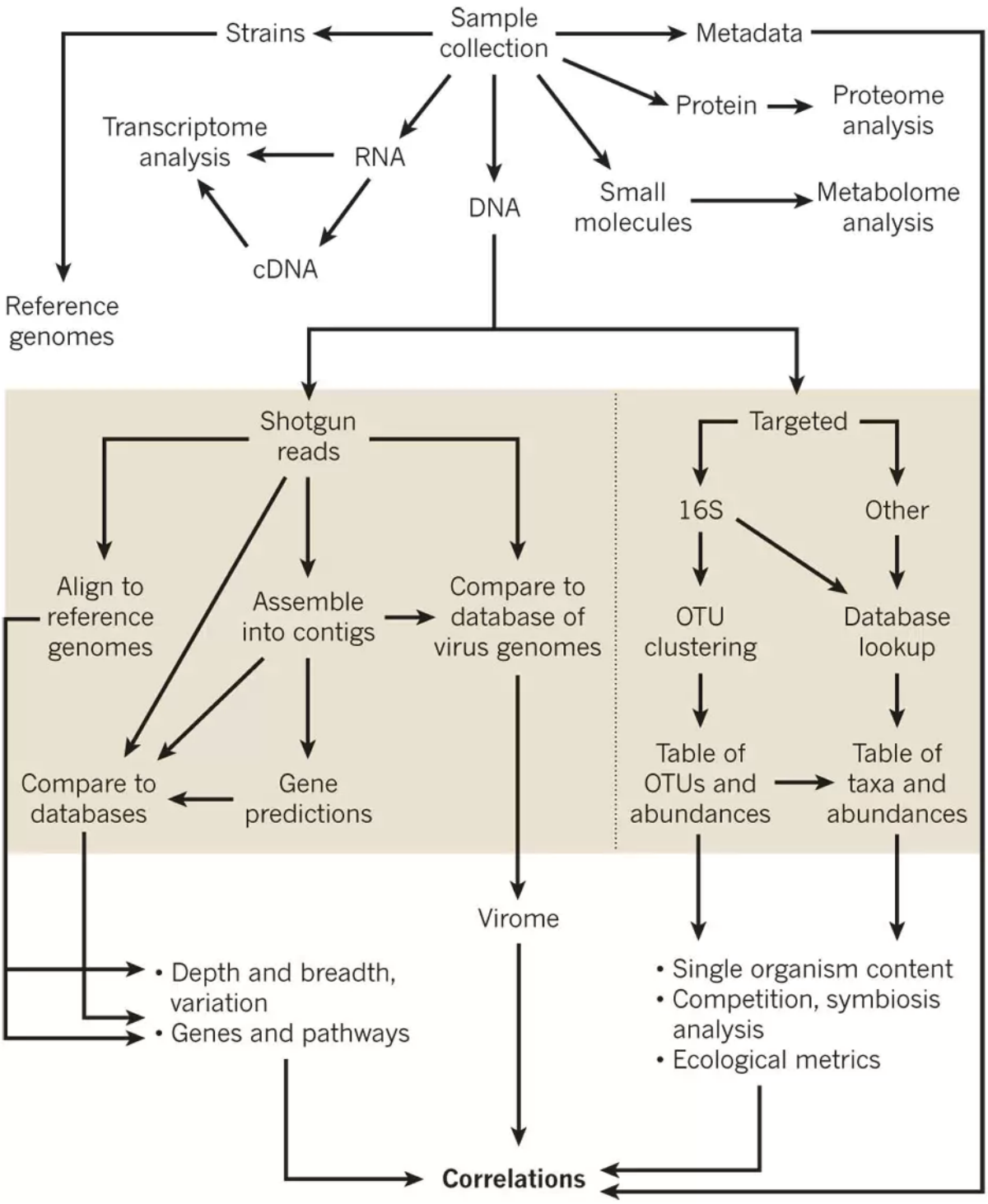


Figure 2-3: Flow diagram to study and analyse the microbial community in an ecosystem. Reproduced from (Weinstock, 2012).

2.7.2 Next generation sequencing: effective and efficient tools to study microbiomes

The automated Sanger method was a classical chain termination DNA sequencing method based on the use of dideoxynucleotides (ddNTPs), and has been used for about 20 years to sequence DNA (Metzker, 2005). Limitations of Sanger's technique led to the search for more convenient and

effective DNA sequencing technologies resulting in the discovery of next-generation sequencing technologies like Roche 454 sequencing and Illumina sequencing (Metzker, 2010) which led to the generation of enormous amounts of DNA sequence data at an unprecedented rate and low cost. Next generation DNA sequencing techniques have been successfully used to study the microbial population structure and function in poultry (Guttala et al., 2017). Traditional culture based methods combined with modern sequencing techniques could be used to develop new probiotics (Adhikari and Kwon, 2017).

However, these high throughput next generation sequencing techniques give shorter read lengths compared to traditional sequencing methods resulting in difficulties with assembling these reads into longer sequences (Morozova and Marra, 2008). The major challenge with these techniques is the downstream data analysis to extract meaningful insights from the vast amount of data (Shendure and Ji, 2008). The current pace of improvement in genomic data analysis and storage lags behind the pace of generating genomic data.

2.7.2.1 Illumina sequencing

Illumina sequencing, which is one of the widely used sequencing by synthesis (SBS) technologies, is a next generation DNA sequencing technology based on reversible dye-terminators which identify the nucleotide bases in DNA during the synthesis process by utilizing a solid (flow cell) surface to immobilize sequencing templates (Quail et al., 2008). During the synthesis of DNA, the base specific fluorescently labelled reversible terminators mixed in the template are attached to each template (Bentley, 2006). The terminator flourophore emits fluorescence which is measured by imaging to identify the added nucleotide followed by removal of the fluorescent group to initiate the next round of the synthesis reactions (Mardis, 2008). The process repeats indefinitely generating a large quantity of sequencing data (Mardis, 2008). Figure 2-4 shows the major steps in the illumina sequencing workflow (Illumina Inc, 2010, Quail et al., 2008, Metzker, 2010, Meyer and Kircher, 2010, Pareek, 2014).

Preparation of DNA library

a. Fragmentation: The genomic DNA library preparation begins with breaking of DNA to produce DNA fragments of 0-1200 base pairs. Although there are different methods to fragment DNA, fragmentation by controllably focused acoustic energy is claimed to be more advantageous than other methods (eg. Nubilization) due to reduced loss of sample fragments. If there is a need for very narrow fragment size distribution, the DNA fragments could be separated on an agarose gel followed by excision from the gel.

b. Adaptor fill-in and size selection: Bst polymerase, an enzyme with strand-displacement property, is used for a fill-in reaction to remove nicks. The reaction is then cleaned up using either solid-phase reversible immobilization (SPRI) or the MinElute PCR Purification Kit depending upon the desired size of DNA fragments to be retained.

c. Indexing PCR and pooling: The template DNAs are amplified, quantified and pooled.

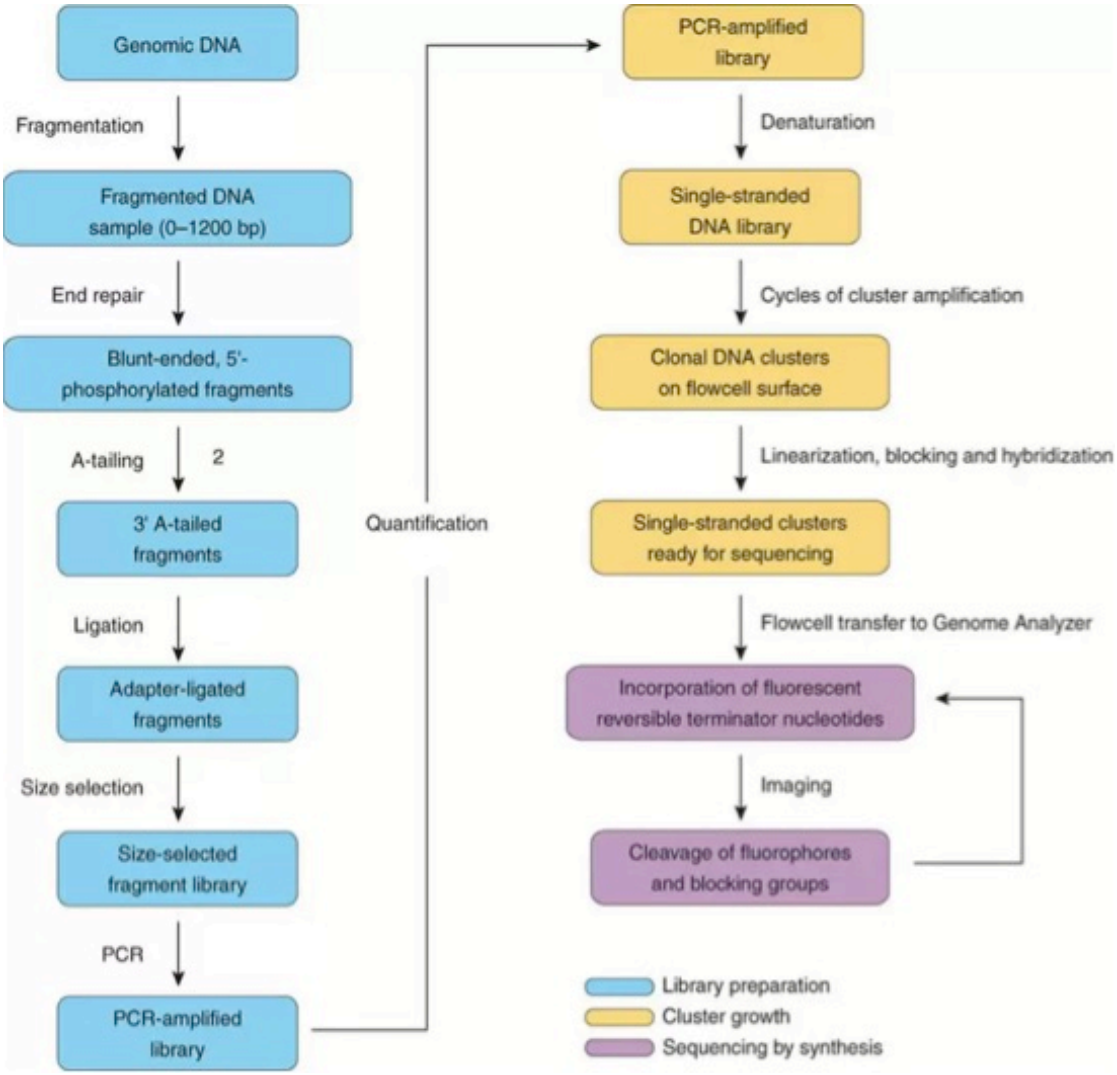


Figure 2-4: Illumina sequencing work-flow. Reproduced from (Quail et al., 2008)

Cluster Generation

a. Immobilization of templates: The templates are immobilized on a flow cell surface which is designed to facilitate the enzyme penetration ensuring stability of DNA templates.

b. Solid phase amplification: The amplification is accomplished in solid support through a PCR containing amplification reagents with immobilized primers and the adaptors. A bridge structure is formed during amplification due to the hybridization of free end of single stranded

templates with the complementary adaptors in the surface of the flow cell. The PCR reactions create a cluster of single stranded DNA templates containing more than 1000 copies of DNA on the surface of the flow cell.

c. Linearization, blocking and hybridization: Following the formation of DNA cluster, the double stranded DNAs in the cluster are converted into single stranded DNA through a process called linearization. This process is followed by the blocking of the 3' ends and hybridization of a sequencing primer.

Sequencing by synthesis

For the actual sequencing process, four fluorescently labelled terminator nucleotides are added in the reaction. When the reaction proceeds, a single deoxynucleoside triphosphate (dNTP) is incorporated in the single stranded template followed by fluorescence imaging to identify the base. The labelled terminator nucleotide then ceases the reaction and the terminator group and the dye is enzymatically cleaved to initiate the next round of reaction (Figure 2-5).

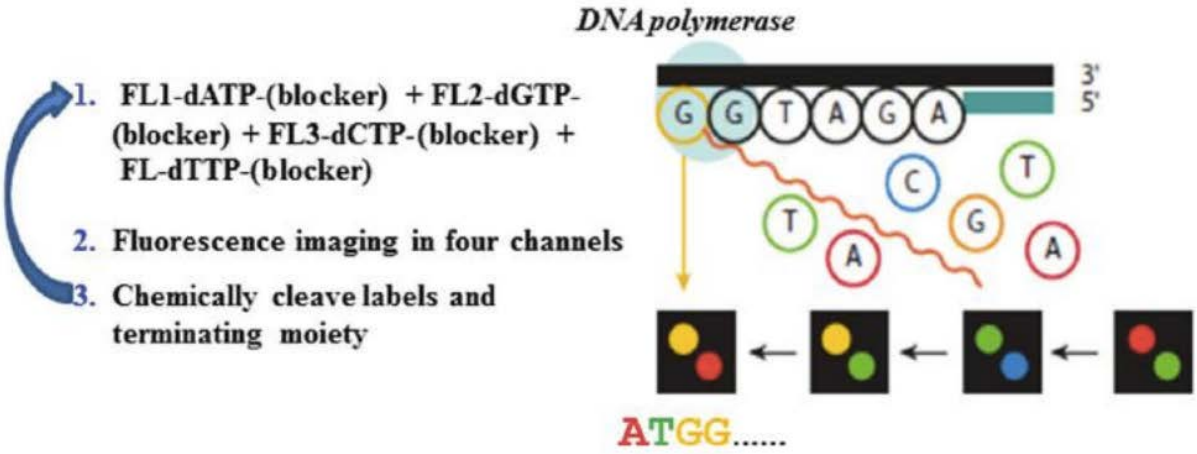


Figure 2-5: Sequencing by synthesis (SBS) reaction chemistry. Reproduced from (Pareek, 2014)

2.7.3 Quantification of microbes in the intestine by real–time quantitative polymerase chain reaction (qPCR)

Real-time quantitate polymerase chain reaction (qPCR) is a quick and robust molecular biotechnology tool which can quantify the copy number of genes simultaneously within the reaction. This technique is very sensitive and specific which makes this technique the most common gene quantification method. Quantitative real time PCR will be used in this study to quantify the population of the probiotic (*B. amyloliquefaciens*) in the content of the chicken GIT and faeces.

The rate of reaction during PCR is not linear as the copies of DNA increase in exponential order at the beginning and eventually will plateau and the reaction will halt once any of the reaction components are exhausted (figure 5). This is the main reason that quantification of DNA copies at the end of the reaction cannot give the correct measurement of DNA number. Therefore, the best point to measure the number of DNA copies is during the exponential phase of the DNA multiplication reaction. Real time qPCR measures the quantity of DNA in the real time while it multiplies and thus makes possible to quantify the DNA during the exponential phase.

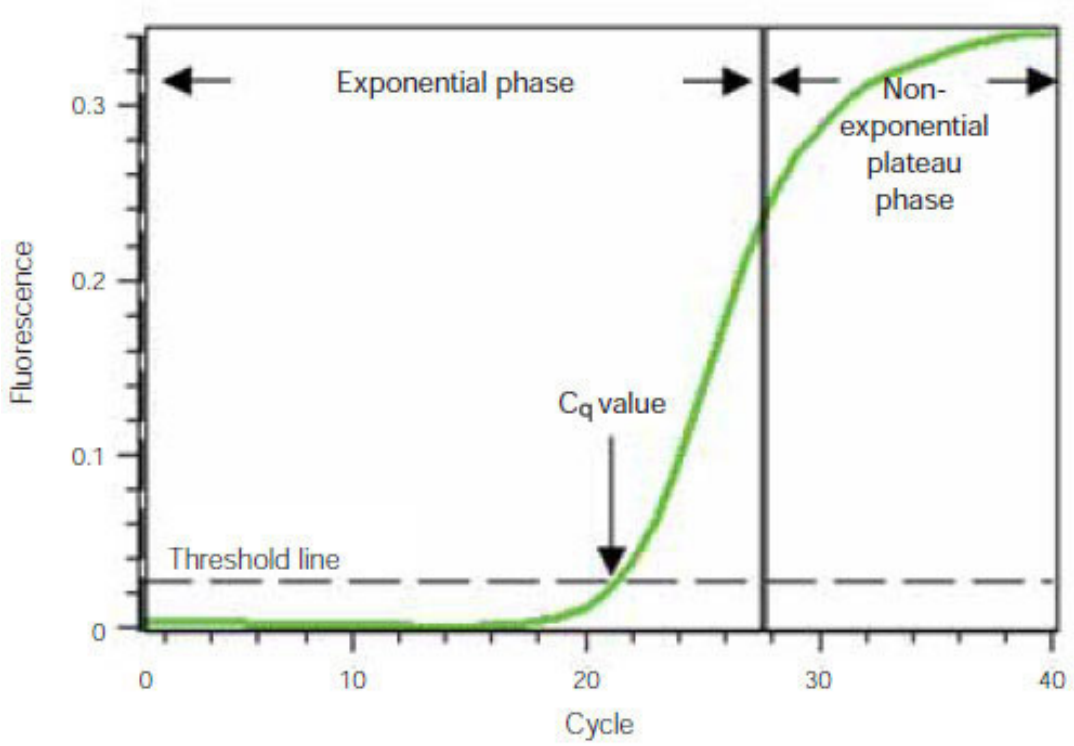


Figure 2-6: Amplification plot of qPCR. Baseline-subtracted fluorescence versus number of PCR cycles. Picture reproduced from www.bio-rad.com

2.7.3.1 Fluorescence as a proxy to quantify DNA

In qPCR, measurement of fluorescence emitted by the particular fluorescent dye attached to the specific nucleotide probe used is converted into the number of copies of DNA in the reaction. For quantification, a target specific oligonucleotide probe is labelled with a fluorescence dye (reporter dye) at the 5' end and a quencher dye at the 3' end. Normally, the probe itself does not emit any detectable fluorescence as the quencher molecule absorbs the fluorescence. In the beginning of the reaction all the three added oligonucleotides present in the reaction, forward primer, reverse primer and dual-labelled probe anneal to the target present in the sample. Taq polymerase in the reaction, which has 5' to 3' exonuclease activity, cleaves the target specific oligonucleotide probe separating reporter dye and quencher dye. While reaction proceeds, the fluorescence is increased with every cycle due to separation of reporter and quencher dyes. During the initial phase of the reaction,

fluorescence from the reporter dye cannot be detected, as this is not significantly detectable from the background fluorescence. With the accumulation of amplification products in the reaction, the amount of free reporter dye keeps on increasing. The cycle number at which the fluorescence in the reaction can be detected as significant from the background fluorescence is called threshold cycle value (Ct or Cq). The more the copies of the template DNA at the beginning of the reaction, the sooner the fluorescence is detected as significantly different and thus fewer cycles are required to reach the threshold point (Bustin et al., 2005). The quantity of DNA in the reaction is quantified by comparing the Ct values of unknown samples to that of a known standard in real time while the reaction proceeds.

2.8 Conclusion

Increasing intensification of poultry with consequent imprudent use of antibiotic growth promoters poses risks to human and animal health in terms of increasing antibiotic resistance in pathogenic microorganisms. Live microorganisms have been studied and used as probiotics for a long time, and as an alternative to antibiotic growth promoters in poultry production. Several probiotics have been found effective in improving performance and preventing disease and the spread of the enteric pathogens.

With the advancement in knowledge in gastrointestinal microbial ecology and mode of action of probiotics, the number of probiotic products available for use in animal nutrition is increasing. However, the microorganisms used as probiotics and their efficacy are highly variable. The claims made by commercial probiotic producers are often difficult to substantiate due to variation in results and lack of understanding about clear mode of action. It is not possible to generalize the mechanism of action of probiotics.

There are many promising effects of probiotics on poultry performance and health. However, the major limitation for the widespread and sustainable use of probiotics is the uncertainty in the reproducibility of effect, with a wide range of probiotic species. Although the use of probiotics could be a potentially viable solution to address the issue of increasing antibiotic resistance, it requires much further study on the effect, mechanism of action and safety of probiotics, to obtain consistent effects and a similar economic benefit to AGPs. Use of next generation DNA sequencing technology to study the effects of probiotics in the structure and function of the GIT microbiota could be a promising method to elucidate the mode of action. As the effects of probiotics in the host is the outcome of interaction between the host and the probiotic microorganism, further studies should be focussed on these interactions to elucidate the mode of action.

Chapter 3 Broiler growth and performance following feed supplementation with Bacillus amyloliquefaciens H57

3.1 Introduction

The current study has been conducted to assess the effects of oral administration of H57 via a mash feed on the growth performance of broiler chickens. Three feeding experiments were conducted using standard animal husbandry practices as approved by the Animal Ethics Committee of the University of Queensland, as required by the Animal Care and Protection Act (2001) and The Australian Code for the Care and Use of Animals for Scientific Purposes.

3.2 Materials and Methods

3.2.1 Culture of Bacillus amyloliquefaciens strain H57

B. amyloliquefaciens strain H57 was cultivated by using a batch fermentation technique (Schofield, 2017). *B. amyloliquefaciens* H57 from stock culture was first multiplied overnight on nutrient agar (about 15 ml in slanting position) in a McCartney bottle at 30°C. McCartney bottle cultures were resuspended with 500 µl of sterile water and the bacterial suspension was then inoculated into 250 mL CELLSTAR® Filter Cap Cell Culture Flask (Greiner Bio-One GmbH, Kremsmünster, Austria). Tissue culture flask flats (with about 120 ml nutrient agar slope) and grown overnight at 30°C. These cultures were then resuspended with about 10 ml of sterile water and the bacterial suspension then inoculated into 500 mL nutrient broth in 1L Erlenmeyer flasks to prepare the starter culture.

One litre of starter culture was inoculated into sterile fermenter medium (Schofield, 2017) prepared in a 20-litre stainless steel modified drum fermenter and incubated for 7 hours at 30°C. The composition of fermenter broth for one drum was 50g Soytone, 20g Yeast Extract, 90g K_2HPO_4, 30g KH_2PO_4, 5g $MgSO_4.7H_2O$, 10g $CaCO_3$ (precipitated), 1g $CaCl_2$, 1g Na_2CO_3, 1g $FeCl_2$, 1g Na_2SO_4, 3g $MnSO_4$, 01 g H_3BO_3, 9 L distilled water and 1 L sugar solution (50 g Glucose + 50g Sucrose). Two 20-litre drum fermenters were used to grow the bacteria aerated initially via a fine metal sparger and at the latter stages of the fermentation with pure oxygen. Foaming in the drums during bacterial growth was reduced by adding 5 ml of 5% (w/v) sterile Antifoam 1920 (Dow Corning, Midland, MI, USA) as required.

After growing bacteria in the drum fermenter for 7 hours, the bacterial suspension (media + bacteria) was transferred into a 100-litre fermenter vessel (Electrolux, Göteborg, Sweden) used for inducing sporulation by nutrient limitation. The fermenter had been filled with 66 L of tap water

mixed with c.50 ml of 5M NaOH to increase the pH to 10. Fermenter media (water) along with all of the attached silicon tubing and accessories were sterilised by heating at 125°C to 128°C for 30 minutes with steam and pressurised with air passed through sterile 0.2 μm air filter (PALL, Cheltenham, VIC, Australia). The bacterial suspension was incubated in the fermenter vessel for about 47 hours with continuous aeration by bubbling sterilised air through a metal sparger (100μ pores) and stirring with a rotating paddle at the bottom of the vessel (Schofield, 2017). The pressure in the fermenter vessel was maintained at 10 kPa by adjusting the inflow and outflow of air into and out of the vessel. The whole process was controlled and monitored through a computerised fermenter control system (Real Time Engineering, Warriewood, NSW, Australia). Excessive foaming was controlled by adding about 10 ml aliquots of 5% (w/v) sterile Antifoam 1920 as required.

The bacterial spores were harvested by using a Sharples G high-speed (15,000rpm) continuous flow barrel centrifuge AS26 (Sharples Separator Works, West Chester, PA, USA). Fermenter content was fed into the centrifuge adjusting the flow rate at 4 L/min until the centrifuge bowl was filled and then at about 0.7 L/min to allow retention of solid materials (bacterial cells and extracellular materials) as much as possible in the centrifuge bowl. After completion of centrifugation, the solid materials in the bowl was collected using a customized spatula (Schofield, 2017).

Finally, the bacterial pellet containing spores and extracellular material, were mixed with an equal quantity (w/w) of feed grade sodium bentonite powder (Unimin Australia Limited, Miles, QLD, Australia) and three volumes of water per unit weight of harvested product (v/w) and mixed with a kitchen food mixer. The resultant paste was lyophilized using Beta 1-8 LSC Laboratory freeze-dryer (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) after freezing the material at -20°C overnight. The freeze-dried inoculum was ground into powder with a mortar and pestle and stored at room temperature.

Concentration of H57 cells (cfu/g) in the inoculum was measured by serially diluting (10-fold) the H57 in bentonite freeze dried inoculum in sterile water and plating the diluted inoculum on nutrient agar plate. The initial dilution bottle containing 1 g of H57 inoculum and 99 ml of sterile water was shaken on a reciprocal shaker for 20 minutes to dislodge the cells before further dilution. The suspension was then serially diluted to count total viable cells while the suspension was heated for 20 minutes at 80°C to kill the vegetative cells and count the spores.

3.2.2 Experimental birds and husbandry practices

All experiments were carried out with different batches of day old male Ross 308 broiler chicks obtained from local hatcheries (one hatchery for experiment 1 and 3 and another hatchery for experiment 2). Birds were fed for 21 days in experiment 1 and 3 and for 35 days in experiment 2. Feed and water were supplied *ad libitum* through one feeder and one drinker in each replicate cage or pen. Birds were fed starter ration till day 14 and grower ration from day 15 to the end of the trial in experiment 1 and 3 and from day 15 to 28 in experiment 2. Finisher diet was fed from day 29 to 35 in experiment 2. The experiments were carried out in environment control rooms with temperature gradually decreased from 32°C on day 1 to 22°C on day 21. Birds were in a 24 hours light regime. Strict hygienic management practices were followed to prevent cross contamination between control and treatment birds with handling of control birds first during feeding, weighing, sampling etc.

In experiment 1, the groups of chicks were housed in cardboard floor pens of size 95cm x 95cm x 65cm (LxWxH) (Figure 3-1) covered with wood shavings with a layer of newspapers with stocking density of 17 birds per square meter. The newspapers were changed weekly.

Figure 3-1 Cardboard floor pens used as replicates for experiment 1 and 2

In experiment 2, half of the chicks (both in control and treatment) were raised in cardboard pens as in experiment 1 and half were raised in cages of size 90cm, 70cm and 50cm (LxWxH) with stocking density of 9 birds per square metre in pens and 13 birds per square meter in cages.

In experiment 3, all of the birds were raised in cages of size 0.85 x 0.7 x 0.5 m (Figure 3-2) high with stocking density of 17 birds per square meter.

Figure 3-2 Wire cages used for experiment 2 and 3

3.2.3 Study design and dietary treatment

In all experiments, day old male chicks were individually weighed and allocated to groups by random stratification so that each group had the same mean and range of bodyweights.

In **experiment 1**, one hundred and eighty day old chicks were randomly allocated into 12 floor pens based on their body weight, resulting in 15 birds in each experimental unit. Cardboard floor pens were then randomly divided into two identical environmentally controlled rooms at the Queensland Animal Science Precinct (QASP), UQ Gatton, with 6 pens in each room. Birds were fed with sorghum and soybean based balanced rations formulated to supply all of the nutrient requirements

of the chicks for two stages of growth: starter and grower (Table 3-1). Chicks in one room received the control diet (E1D1 – starter and E1D1 – grower where E1D1stands for Experiment 1 Diet 1) while chicks in the other room received the same diet but mixed with 2×10^7 spores of *B. amyloliquefaciens* strain H57 (H57) per gram of feed (E1D1 – starter +H57 and E1D1 – grower +H57) both in the starter and grower diets. Starter diet was fed up to 14 days and grower diet was fed from 15 to 21 days. Thus, there were two experimental diets (with and without inoculum of *B. amyloliquefaciens* strain H57). One extra control pen was kept in the inoculation treatment room to test for cross contamination between control and treatment pens. This pen was not included for other performance measurement and analysis. Transfer of H57 from H57 treated groups to control chickens in the same room was tested by counting H57 in the GIT of control chickens kept in the H57 room by quantitative PCR as described in chapter 6. There was no cross contamination detected within the room (data not shown) but H57 was detected in the GIT of treated birds.

Table 3-1 Composition of starter and grower diet (E1D1 – starter and E1D1 - grower) in experiment 1

Ingredients	E1D1 - Starter (%)	E1D1 – Grower (%)
Sorghum	54.72	59.52
Soybean Meal	32.9	27.8
Canola meal	3.2	3
Meat and Bone Meal	4.4	3.3
Sun-soy oil	2.94	4.33
Lysine.HCl 78	0.24	0.22
DL Methionine	0.37	0.33
L-Threonine	0.1	0.09
Limestone fine	0.063	0.25
MDCP Biophos	0.114	0.181
Salt fine	0.23	0.24
Sodium bicarbonate	0.2	0.16
Vitamin & minerals premix[2]	0.5	0.5
Choline chloride	0.05	0.06

In **experiment 2**, 190 two-day-old male chicks were randomly divided into 12 cardboard pens and 12 cages based on body weight as above, resulting in 8 birds per replicate. As we planned to conduct future experiments with cages, half of the birds were kept in cages to compare the effects of H57 in pens with that in cages. Six pens and six cages were kept in one temperature controlled room at QASP, while the remaining six pens and six cages were placed in adjacent room. This was

[2] Vit A: 10000000IU; Vit D_3: 2500000IU; Vit E: 30g; Vit K_3: 2g; Vit B_1: 1.5g; Vit B_2: 8g; Vit B_6: 4g; Vit B_{12}: 20mg; D-Calcium pantothenate: 15g; Folic acid: 2g; Nicotinic acid: 45g; Biotin: 135mg; Co: 200mg; Cu:6g; Fe: 50g; I: 750mg; Mn: 75g; Mo: 1g; Se: 150mg; Zn:60g

a different and larger room to those used in Experiment 1. Control and treatment rooms were identical and environmentally controlled. Birds in the control room were fed a wheat and soybean based control diet (E2D1 – starter, E2D1 – grower and E2D1 – finisher; Table 3-2) while birds in the treatment room were supplied with the same diet mixed with 2×10^7 spores of *B. amyloliquefaciens* strain H57 per gram of feed (E2D1 – starter + H57, E2D1 – grower + H57 and E2D1 – finisher + H57). Birds were fed the starter diet for 14 days, with the grower diet from 15 to 28 days and the finisher diet from 29 to 35 days. Hence, treatments were similar to those in experiment 1 (diet with and without probiotic) but based on wheat and soybean in contrast to sorghum and soybean in experiment 1.

Table 3-2: Composition of starter, grower and finisher diet in experiment 2

Ingredients	E2D1 (Starter-diet) (%)	E2D2 (Grower-diet) (%)	E2D3 (Finisher-diet) (%)
Wheat fine 2 (phantom)	64.88	69.89	71.63
Oil	2.6	3.6	3.93
Soybean meal 48	28.7	22.9	19.18
Limestone fine bags	1.27	1.4	1.33
Ram dried fine no2 salt	0.2	0.17	0.2
Sodium bicarb	0.27	0.2	0.13
Choline chloride 70%	0.03	0.03	0.03
Dl-methionine 58.1	0.31	0.26	0.21
L-lysine 94.5	0.34	0.31	0.25
L-threonine 73.7	0.13	0.12	0.09
Biofos mdcp bags	0.9	0.75	0.65
Rap broil start/grow pmx	0.2	0.2	0.2
Rovabio maxima 10% pmx broil (phantom)	0.05	0.05	0.05
Bentonite (control)/H57 inoculum in treatment group (concentration $1.67\text{x}10^{10}$ cfu/g)	0.12	0.12	0.12
Celite	-	-	2

In **Experiment 3**, 432 day old male chicks were allocated into 36 cages based on body weight resulting in 12 chicks in each cage. The dietary treatments were; 1) Sorghum based control diet (E3D1), 2) Wheat based control diet (E3D2), 3) Sorghum + Wheat blended control diet (E3D3), 4) E3D1 + H57, 5) E3D2 + H57 and 6) E3D3 + H57. Soybean was the major source of protein in all diets. The H57 inoculum added to each inoculated treatment diet was 8.33×10^7 per gram for starter diets and 5.19×10^7 per gram for grower diets. These concentrations were designed to provide uptake by the birds of $>10^9$ cells of H57 per bird per day. The ingredients and composition of starter and grower diets are presented in Table 3-3. Experiment 3 was carried out in single room as cross contamination test from experiment 1 showed no cross contamination from H57 groups to control. Eighteen control cages were kept at one end of a large temperature controlled room (13.5m

× 3.9m) and 18 treatment cages were placed at the other end of the room to avoid the cross contamination among control and treatment cages. Three control diets were allocated randomly to the 18 control cages and 3 H57 inoculated diets were allocated randomly to the other 18 or treatment cages.

No other enzymes, anticoccidials or antimicrobials were added to the diet because the aim was to examine the effect of H57 *per se* on bird performance. H57 may have properties similar to such additives and this might not have been demonstrated in their presence.

Table 3-3: Composition of starter and grower diets in experiment 3

Ingredient	E3D1-Starter (%)	E3D1-Grower (%)	E3D2-Starter (%)	E3D2-Grower (%)	E3D3-Starter (%)	E3D3-Grower (%)
Wheat	0	0	52.83	58.30	25.96	28.98
Sorghum	52.82	56.64	0	0	28.22	29.24
SBM	34.65	30.17	36.45	27.38	32.49	27.88
Canola Meal	3.00	3.00	1.00	3.00	3.00	3.00
Meat and Bone Meal 50% Beef Aust	4.69	4.00	0.90	2.30	4.69	3.80
Soy Oil	3.12	4.59	5.54	6.73	3.87	5.46
Lysine.HCl 78 Aust	0.18	0.13	0.18	0.16	0.20	0.15
DL Methionine	0.35	0.30	0.29	0.24	0.32	0.27
L-Threonine 98% Aust	0.07	0.05	0.07	0.05	0.08	0.06
Limestone Fine	0	0.10	0.70	0.45	0.05	0.15
MDCP Biophos Aust 15/21	0.02	0	0.87	0.32	0	
Salt Fine	0.24	0.27	0.27	0.21	0.21	0.23
Sodium Bicarb	0.17	0.11	0.22	0.24	0.22	0.17
Generic Vitamin & Minerals Pmx	0.50	0.50	0.50	0.50	0.50	0.50
Choline Chloride 60% Dry	0.05	0.06	0.05	0.04	0.05	0.05
Bentonite (control)/H57 inoculum in treatment group (concentration $6.1x10^{10}$ cfu/g)	0.14	0.08	0.14	0.08	0.14	0.08

3.2.3.1 Preparation of experimental diets

The probiotic H57 was supplied as a freeze dried preparation in a bentonite carrier. The required quantity of inoculum powder was mixed with approximately 2 kg of ground sorghum or ground wheat (based on diet) in a blender and then further diluted to a 10% mixture with the ground cereal.

All of the ingredients of the diet were weighed and mixed in a feed mixer. The inoculum mix, prepared as above, was added and the feed was mixed again. Feed was mixed during the week before the start of each experiment and stored at room temperature till fed as a mash.

3.2.4 Performance data collection

Weights of chicks were measured weekly using a flat top weighing balance. Feed intake was also measured weekly. Mortality was recorded with cage or pen number and weight of dead birds.

Digesta samples from the ileum and caeca and tissue samples of duodenum, jejunum, ileum and caeca from one bird per replicate were collected at day 4, day 13 and day 21 for microbial and molecular/genomic studies. The samples were immediately frozen in liquid nitrogen and transported in dry ice to store at -80°C. Similarly, faecal samples were also collected at day 4, day 13 and day 21.

3.2.5 Statistical analysis

Means of body weight, daily weight gain, daily feed intake and feed conversion ratio (FCR) in individual cages or pens were analysed by one-way ANOVA with a p-value of <0.05 denoting a significant difference. Data were adjusted for any mortalities. The normality of distribution and homogeneity of variances were confirmed before applying the statistical tests. Tukey-Kramer post hoc test was used for pairwise comparison of means.

3.3 Results

3.3.1 Production of *B. amyloliquefaciens* H57 inoculum

Approximately 10^{13} spores of H57 (average of 6 batches) per 100l fermenter run batch were produced. The details of the quantity of H57 production from each batch of fermenter runs with quantity of wet pellet, dry H57-bentonite inoculum powder and concentration of H57 is presented elsewhere (Schofield, 2017).

3.3.2 Bird Performance - Experiment 1

The results for Experiment 1 are shown in Table 3-4. The average daily weight gain of the birds fed H57 was significantly greater than controls over the periods of day 0 to 14 (7.0%) and day 0 to 21 (6.9%). This resulted in significantly higher body weight of birds fed H57 at day 21 (896 g vs 845 g) when compared to control birds. Interestingly, there were no differences in feed intake between treatments but dietary addition of H57 significantly improved FCR.

Table 3-4: Effects of *Bacillus amyloliquefaciens* strain H57 on performance of broiler chicken fed sorghum based diet (experiment 1)

Variables[#]	Days (post hatch)	Dietary Treatment		p-value
		E1D1 (control)	E1D1+H57	
BW (g per bird)	Initial	38.1	38.1	0.570
	day 7	165.2	169.3	0.248
	day 14	448.3[a]	477.7[b]	0.05
	day 21	844.7[a]	895.9[b]	0.007
Average daily weight gain (g)	day 0 to 7	18.2	18.8	0.223
	day 8 to 14	40.5[a]	44.0[b]	0.007
	day 0 to 14	28.4[a]	30.4[b]	0.004
	day 15 to 21	56.2	59.4	0.075
	day 0 to 21	34.8[a]	37.2[b]	0.002
Average daily feed intake (g)	day 0 to 7	18.2	18.0	0.82
	day 8 to 14	55.0	53.3	0.541
	day 0 to 14	35.1	34.3	0.565
	day 15 to 21	85.0	86.8	0.305
	day 0 to 21	48.8	48.9	0.943
Feed conversion ratio	day 0 to 7	1.00	0.96	0.151
	day 8 to 14	1.36[a]	1.21[b]	0.024
	day 0 to 14	1.23[a]	1.13[b]	0.027
	day 15 to 21	1.51	1.46	0.163
	day 0 to 21	1.35[a]	1.27[b]	0.022

[a, b] Means within columns followed by different superscripts are significantly different at $P < 0.05$

3.3.3 Bird Performance - Experiment 2

The results for Experiment 2 are shown in Table 3-5. There was no significant difference in body weight between treatments during the study, with no significant difference in ADG between control and treated birds overall (day 0 to 35). In this experiment, H57 treated birds ate significantly less feed (c. 4%, 95.8g/d vs 92g/d) compared to control birds during the 35 days of the trial. However, there was no effect of H57 on FCR.

Table 3-5: Effects of *Bacillus amyloliquefaciens* strain H57 on performance of broiler chickens fed a wheat based diet (experiment 2)

Variables[#]	**Days (post hatch)**	**Dietary Treatment**		**p-value**
		E2D1	**E2D1+H57**	
BW (g)	Initial	44.1	44.1	0.915
	day 7	185.9	185.7	0.943
	day 14	482.2	480.9	0.868
	day 21	963.4	941.7	0.186
	day 28	1641.1	1592.6	0.070
	day 35	2335.6	2298.2	0.102
ADG (g)	day 0 to 7	20.3	20.2	0.952

	day 8 to 14	42.3	42.2	0.848
	day 0 to 14	31.3	31.2	0.870
	day 15 to 21	68.8[a]	65.8[b]	0.041
	day 22 to 28	94.6	92.6	0.513
	day 29 to 35	99.2[a]	89.4[b]	0.018
	day 0 to 35	64.8	61.8	0.058
ADFI (g)	day 0 to 7	21.2	20.9	0.574
	day 8 to 14	56.3	54.6	0.273
	day 0 to 14	38.8	37.7	0.301
	day 15 to 21	85.2[a]	79.2[b]	0.001
	day 22 to 28	142.9	141.3	0.524
	day 29 to 35	175.8[a]	167.5[b]	0.049
	day 0 to 35	95.8[a]	92.0[b]	0.019
FCR	day 0 to 7	1.05	1.03	0.486
	day 8 to 14	1.25	1.25	0.811
	day 0 to 14	1.24	1.21	0.331
	day 15 to 21	1.24	1.21	0.404
	day 22 to 28	1.52	1.56	0.487
	day 29 to 35	1.79	1.89	0.064
	day 0 to 35	1.49	1.50	0.592

[a, b] Means within columns followed by different superscripts are significantly different at $P < 0.05$ (highlighted rows)

3.3.4 Bird Performance - Experiment 3

The results for Experiment 3 are shown in Table 3-6. In this experiment, H57 had a significant effect on growth rate of broilers. For the sorghum based diet, H57 improved the average daily weight gain from day 0 to 21 from 39.6 g/day (control) to 46.6 g/day (H57 birds) or some 18% (Figure 3-4a and Figure 3-3). Average weight of birds fed the H57 treatment were 184 g at day 7, 506 g at day 14 and 1027 g with corresponding values for control birds 152 g, 411 g and 922g, respectively.

The growth rate of birds fed the wheat based diet supplemented with H57was also improved; with an overall difference of 8.4% (40.5 g/day/bird vs 43.9 g/day/bird) between control and treated birds from day 0 to 21 (Figure 3-4b and Figure 3-3). The average weight of H57 treated birds were 183 g at day 7, 483 g at day 14 and 994 g at day 14 with corresponding weights of control birds of 158 g, 416 g and 930 g, respectively

In contrast, there were no significant differences for growth rate and body weight between control and H57 treated birds fed the diet based on a mixture of sorghum and wheat (Figure 3-4c and Figure 3-3). Growth rate and body weight of birds on the combined sorghum and wheat based diets (both H57 and control) were similar to those of birds fed on sorghum or wheat based diets supplemented with H57.

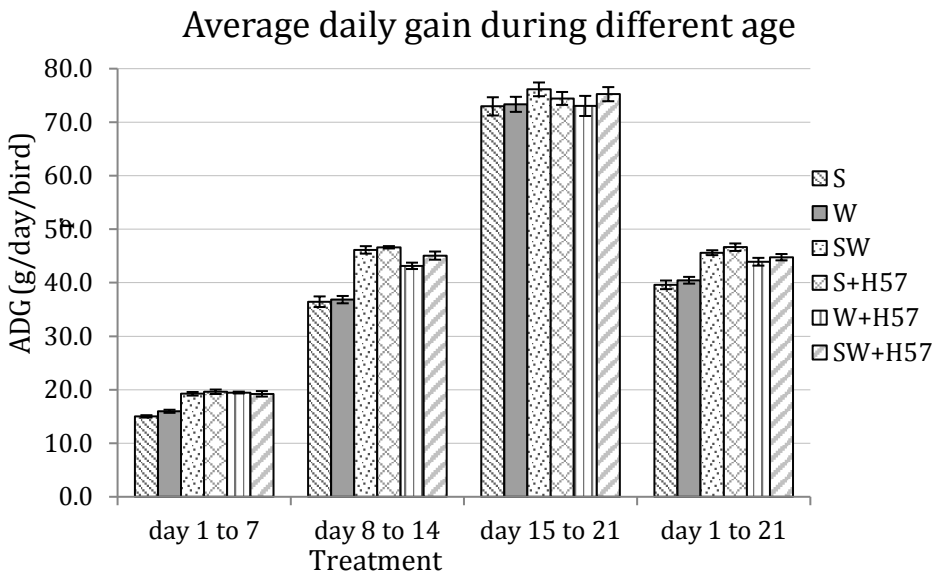
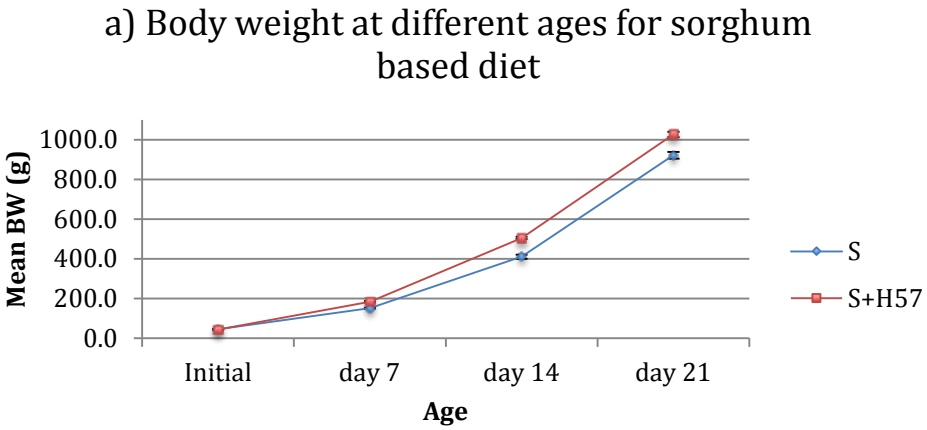
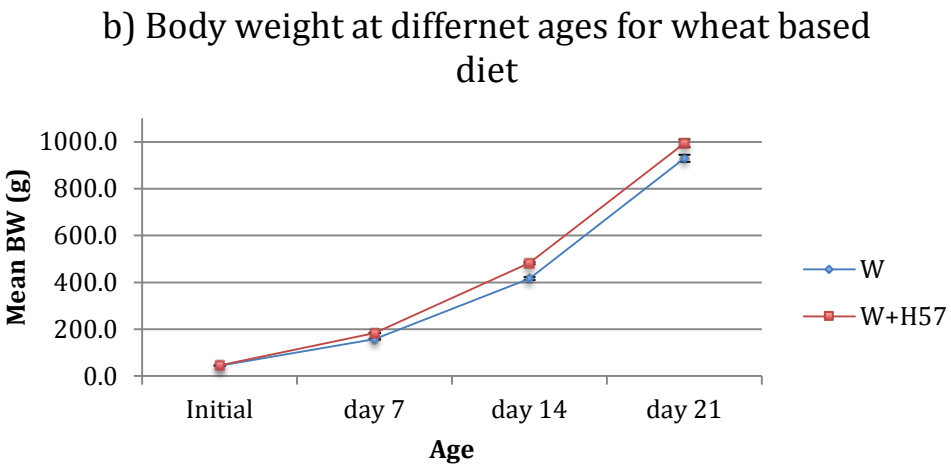


Figure 3-3: Comparison of average daily gain among different treatments at different ages in experiment 3. Error bars show standard error of the mean. S = Sorghum based feed. W = Wheat based feed, SW = Sorghum and wheat blend feed, S+H57 = Sorghum based diet mixed with Bacillus amyloliquefaciens strain H57, W+H57 = Wheat based diet mixed with H57, SW+H57 = Sorghum and wheat blend diet mixed with H57.





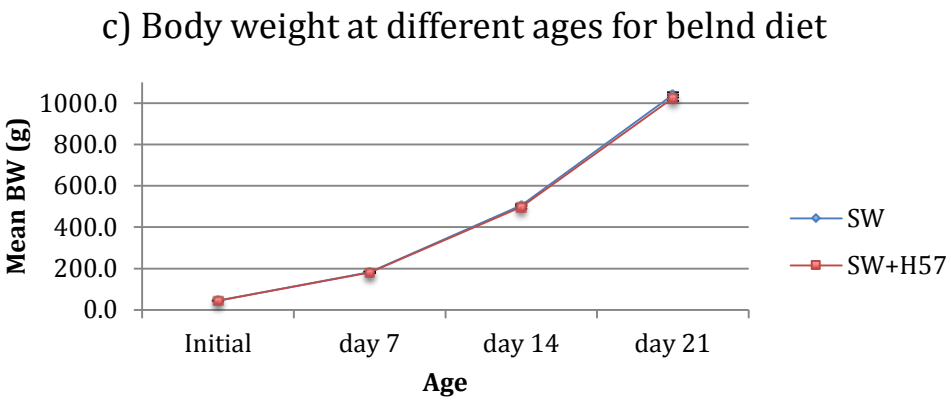


Figure 3-4: Body weight of chicks fed different diet types at different ages in experiment 3. Error bars show standard error of mean. a) Body weight of chicks fed sorghum based diet, b) body weight of chicks fed wheat based diet, c) body weight of chicks fed sorghum and wheat blend diet. S = Sorghum based feed. W = Wheat based feed, SW = Sorghum and wheat blend feed, S+H57 = Sorghum based diet mixed with *Bacillus amyloliquefaciens* strain H57, W+H57 = Wheat based diet mixed with H57, SW+H57 = Sorghum and wheat blend diet mixed with H57.

Table 3-6: Effects of *Bacillus amyloliquefaciens* strain H57 on performance of broiler chicken fed different diets (experiment 3)

Variables[#]	Days (post hatch)	Dietary Treatment						Statistics	
		E3D1 (Sorghum)	E3D2 (Wheat)	E3D3 (Sorg+Wheat)	E3D1+H57	E3D2+H57	E3D3+H57	F* (5, 30)	p-value
BW (g)	Initial	44.9 ± 0.07	44.8 ± 0.15	44.8 ± 0.16	44.8 ± 0.09	45.2 ± 0.14	45.1 ± 0.17	1.659	> 0.175
	day 7	151.9^{a} ± 2.10	157.6^{a} ± 2.04	183.1^{b} ± 2.42	184.0^{b} ± 2.97	183.7^{b} ± 1.77	181.0^{b} ± 3.65	32.675	< 0.001
	day 14	411.1^{a} ± 10.35	416.4^{a} ± 5.82	506.2^{b} ± 5.69	505.8^{b} ± 5.61	482.5^{b} ± 4.98	497.7^{b} ± 8.63	38.857	< 0.001
	day 21	921.8^{a} ± 16.30	929.7^{a} ± 15.37	1039.2^{b} ± 12.22	1026.9^{b} ± 13.76	993.7^{b} ± 16.32	1024.4^{b} ± 14.59	12.051	< 0.001
ADG (g)	day 0 to 7	15.0^{a} ± 0.22	16.0^{a} ± 0.29	19.3^{b} ± 0.34	19.6^{b} ± 0.42	19.5^{b} ± 0.17	19.2^{b} ± 0.51	35.322	< 0.001
	day 8 to 14	36.5^{a} ± 0.99	36.8^{a} ± 0.69	46.1^{cd} ± 0.70	46.6^{bc} ± 0.22	43.2^{de} ± 0.60	45.1 ± 0.76^{cd}	43.342	< 0.001
	day 0 to 14	25.0^{a} ± 0.60	25.4^{a} ± 0.39	32.2^{b} ± 0.20	32.2^{b} ± 0.20	30.2^{c} ± 0.33	30.6^{bc} ± 0.71	53.381	< 0.001
	day 15 to 21	73.0 ± 1.70	73.3 ± 1.41	76.1 ± 1.28	74.4 ± 1.21	73.0 ± 1.88	75.2 ± 1.32	0.773	> 0.577
	day 0 to 21	39.6^{a} ± 0.80	40.5^{a} ± 0.63	45.6^{b} ± 0.46	46.6^{b} ± 0.70	43.9^{b} ± 0.73	44.8^{b} ± 0.59	18.525	< 0.001
ADFI (g)	day 0 to 7	17.7^{a} ± 0.23	18.4^{a} ± 0.22	19.9^{b} ± 0.38	20.9^{b} ± 0.24	20.8^{b} ± 0.16	20.5^{b} ± 0.22	29.659	< 0.001
	day 8 to 14	47.3^{a} ± 1.35	49.6^{a} ± 0.85	56.4^{b} ± 0.54	57.1^{b} ± 1.31	55.3^{b} ± 0.56	56.9^{b} ± 1.32	16.248	< 0.001
	day 0 to 14	31.4^{a} ± 0.70	32.7^{a} ± 0.46	36.7^{b} ± 0.40	37.8^{b} ± 0.41	36.6^{b} ± 0.30	36.7^{b} ± 0.44	31.302	< 0.001
	day 15 to 21	91.3^{ab} ± 1.39	94.8^{bc} ± 1.33	102.8^{de} ± 1.83	101.5^{de} ± 1.53	97.2^{bd} ± 1.17	99.1^{cd} ± 0.92	9.525	< 0.001
	day 0 to 21	48.9^{a} ± 0.77	50.9^{a} ± 0.65	56.0^{b} ± 0.58	56.4^{b} ± 0.50	54.0^{b} ± 0.53	54.9^{b} ± 0.53	24.822	< 0.001
FCR	day 0 to 7	1.18^{a} ± 0.02	1.15^{a} ± 0.01	1.04^{b} ± 0.02	1.06^{b} ± 0.02	1.07^{b} ± 0.01	1.07^{b} ± 0.02	10.065	< 0.001
	day 8 to 14	1.30^{ab} ± 0.03	1.35^{ac} ± 0.03	1.22^{bd} ± 0.01	1.23^{bd} ± 0.03	1.28^{ab} ± 0.02	1.26^{ab} ± 0.03	3.407	< 0.05
	day 0 to 14	1.26^{ab} ± 0.02	1.28^{bc} ± 0.02	1.16^{de} ± 0.01	1.17^{de} ± 0.02	1.21^{ad} ± 0.02	1.20^{ad} ± 0.02	6.832	< 0.001
	day 15 to 21	1.25^{a} ± 0.03	1.29^{ab} ± 0.02	1.35^{bc} ± 0.01	1.34^{ac} ± 0.02	1.33^{ac} ± 0.03	1.34^{ac} ± 0.02	3.286	< 0.05
	day 0 to 21	1.26 ± 0.02	1.29 ± 0.02	1.26 ± 0.01	1.26 ± 0.01	1.27 ± 0.02	1.27 ± 0.01	0.708	> 0.622

[#]measurements are presented as group mean ± standard error of mean (SEM).
[a, b, c, d, e] numbers in the same row with different superscript letters are significantly different at p<0.05 (i.e. numbers with at least one common superscript are not statistically different).
*numbers in the brackets are degree of freedom between groups and degree of freedom among groups
BW = body weight. ADG = average daily gain. ADFI = average daily feed intake. FCR = feed conversion ratio.
E3D1 = experiment 3, diet 1 (diet based on sorghum). E3D2 = experiment 3, diet 2 (diet based on wheat). E3D3 = experiment 3, diet 3 (diet based on mixture of sorghum and wheat).

In experiment 3, feed intake was significantly increased when H57 was mixed with both the sorghum and wheat based diets (Table 3-6 and Figure 3-5). In the sorghum based diet, average daily feed intake increased throughout the 21 days of the trial by 15.3% (from 48.9 g/day/control bird to 56.4 g/day/H57 bird). The increase in feed intake for wheat fed birds for the same period was 6.1% (from 50.9 g/day/control bird to 54 g/day/H57bird). In contrast there was no improvement in feed intake for birds fed the wheat and sorghum mix supplemented with H57.

Feed use efficiency was improved by H57 from day 0 to 7 with a reduction in the FCR from 1.18 to 1.06 (9.5%) in sorghum based diet and from 1.15 to 1.07 (7.0%) in wheat based diet (Table 3-6 and Figure 3-6). However, this effect did not last beyond day 7 and there was no significant difference overall from day 0 to 21 for both diets. There was no effect of H57 on FCR for the sorghum and wheat blended diet.

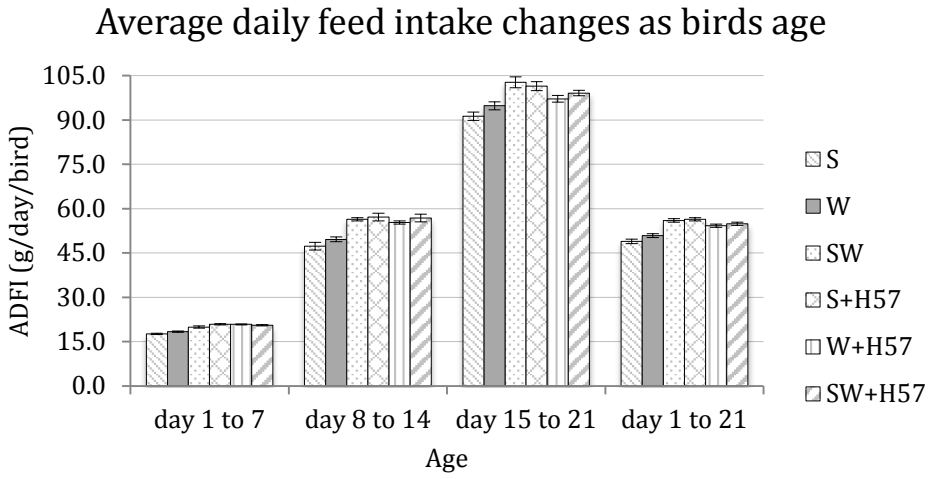


Figure 3-5: Comparison of average daily feed intake among different treatments at different ages in experiment 3. Error bars show standard error +/- of the mean. S = Sorghum based feed. W = Wheat based feed, SW = Sorghum and wheat blend feed, S+H57 = Sorghum based diet mixed with *Bacillus amyloliquefaciens* strain H57, W+H57 = Wheat based diet mixed with H57, SW+H57 = Sorghum and wheat blend diet mixed with H57.

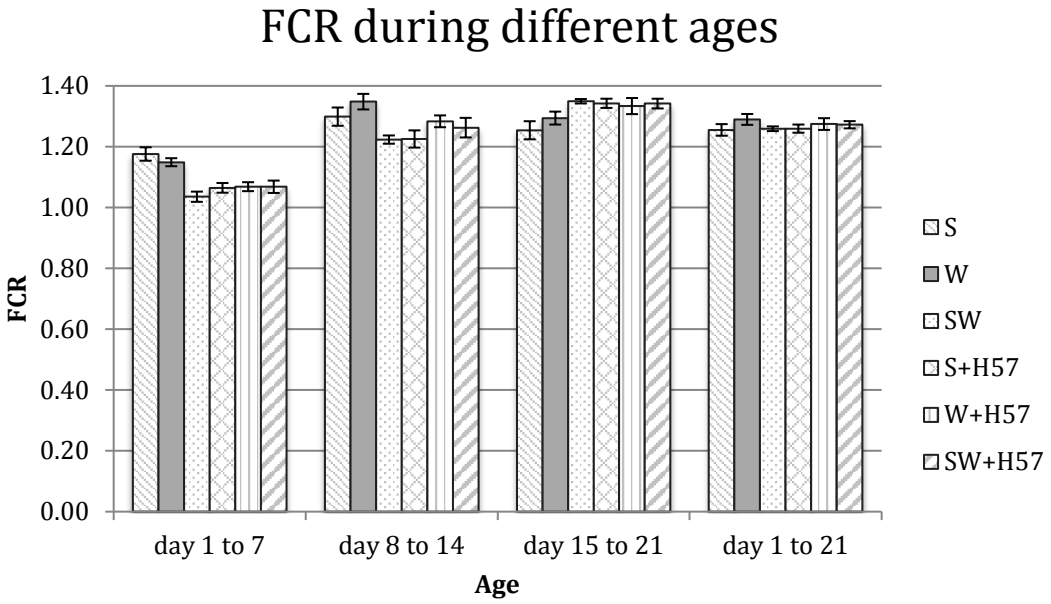


Figure 3-6: Comparison of feed conversion ration (FCR) among different treatments at different ages in *experiment 3*. Error bars show standard error of mean. S = Sorghum based feed. W = Wheat based feed, SW = Sorghum and wheat blend feed, S+H57 = Sorghum based diet mixed with *Bacillus amyloliquefaciens* strain H57, W+H57 = Wheat based diet mixed with H57, SW+H57 = Sorghum and wheat blend diet mixed with H57.

3.4 Discussion

Sorghum is one of the major energy sources used in poultry diets in Australia. This grain, however, has major limitations when used as sole source of carbohydrate due to the presence of anti-nutritional factors such as kafirin, phytate, and polyphenolic compounds (Bryden et al 2009). Therefore, the first experiment was undertaken with the feed based on this challenging ingredient as the energy source. After encouraging results in terms of body weight gain and food conversion ratio the trial was repeated with the diet based on wheat, another common carbohydrate source used in the poultry industry. The third experiment was then designed with three diet types, sorghum, wheat and a sorghum and wheat blend, in order to assess whether there was an interaction between the type of cereal grain in the diet and the response to the probiotic. Soybean was the main protein source in all of the diet types.

The first experiment was conducted for 21 days as raising chicks for 21 days while conducting chicken growth experiments is common practice. This is because if a treatment has positive effects on growth, the effect should generally be obvious by 21 days. This study was the first phase of research assessing the effects of H57 as a probiotic in poultry and the experiment was conducted for 21 days at the beginning to establish any effects. The second experiment was then conducted for 35 days. Unexpectedly, the result of the second experiment didn't match with the first. Therefore, the third experiment was also conducted for 21 days with more variables. Moreover, It has been

suggested that maturity of the chicken gastrointestinal tract microbiome occurs between15 and 22 days of age (Ranjitkar et al., 2016)

Although there were significant positive effects of H57 on broiler growth rate and feed conversion, the effects were not consistent. Birds fed sorghum based diets supplemented with H57 had improved body weight gain and feed conversion both in experiments 1 and 3. The response to wheat based diets varied between experiments, as there was a positive effect of H57 in experiment 3 but not in experiment 2 when the wheat based diet was supplemented with H57. In contrast, no improvement in bird performance was observed when the sorghum and wheat blended diet was supplemented with H57 in experiment 3. In contrast to our result, Crisol-Martinez *et al.* (2017) reported that chicks with sorghum-based diet had better growth performance than chicks with sorghum and wheat mixed diet.

The major objectives of this PhD thesis were to analyze the impacts of the probiotic *Bacillus amyloliquefaciens* H57 on performance and intestinal microbial population and microbial functions across a range of feed compositions. In all of the experiments (Ex. 1, 2 and 3) the only variable that is different between control and treatment diets is the H57 added to the treatment diet. In each experiment, both control and treatment diets were prepared using the same batch of the ingredients, prepared on the same day using the same method and equipment. Significance of differences in performance, abundance of particular microbes (OTUs) and abundance of genes responsible to encode particular functions were analyzed by comparing the data between control and treatment (H57) groups for each experiment. Diet for each experiment was prepared with different batches of ingredients. Analysis of diets to measure nutrient composition, anti-nutritional factors and soluble non-starch polysaccharides was not undertaken, as it would require an expansion of the experimental variables tested beyond the scope of this PhD.

There do not appear to be any other studies of the effect of feeding *B. amlyloliquefaciens* on the performance of poultry fed sorghum and/or wheat based diets. Nevertheless, our results are in partial agreement with Ahmed et al. (2014) and Lei et al. (2015) who reported positive effects on body weight gain, feed intake and FCR of broiler chickens fed a corn and soybean based diet supplemented *B. amyloliquefaciens*. In earlier studies, broilers fed with a commercial probiotic product (Ecobiol) containing *B. amyloliquefaciens* had increased growth rate (Ortiz et al., 2013) and improved feed efficiency (Diaz, 2007). However, it is difficult to compare studies as different basal diets were fed and the amount of *B.amyloliquefaciens* added to the diet is not given. In the current study, the aim was for each bird to have an intake of 10^9 cells/day.

The mechanism of action of H57 that results in the improved growth rate of birds is not apparent at this point in time. However, there were some common features across all experiments, which may assist in determining the mode of action. As stated earlier, there was no difference in growth rate and feed conversion between birds fed diet with and without H57 in experiment 2 (wheat based diet) and in experiment 3 (sorghum and wheat mixed diet). However, birds (both control and H57) in these experiments had what would be estimated to be the optimum growth rate (Aviagen, 2014b). To illustrate this, expected average daily gain (performance objective) and actual daily gain are shown in Figure 3-8 and Figure 3-9c. In contrast, growth rate of the control group was lower than predicted in experiment 1 (Figure 3-7), experiment 3 with the sorghum based diet (Figure 3-9a) and experiment 3 with the wheat based diet (Figure 3-9b). Interestingly, the birds fed the diet with H57 had significantly higher growth rates than those fed the Control diet in these experiments. Therefore, one possible mechanism of action of H57 could be overcoming a suppression in growth rate.

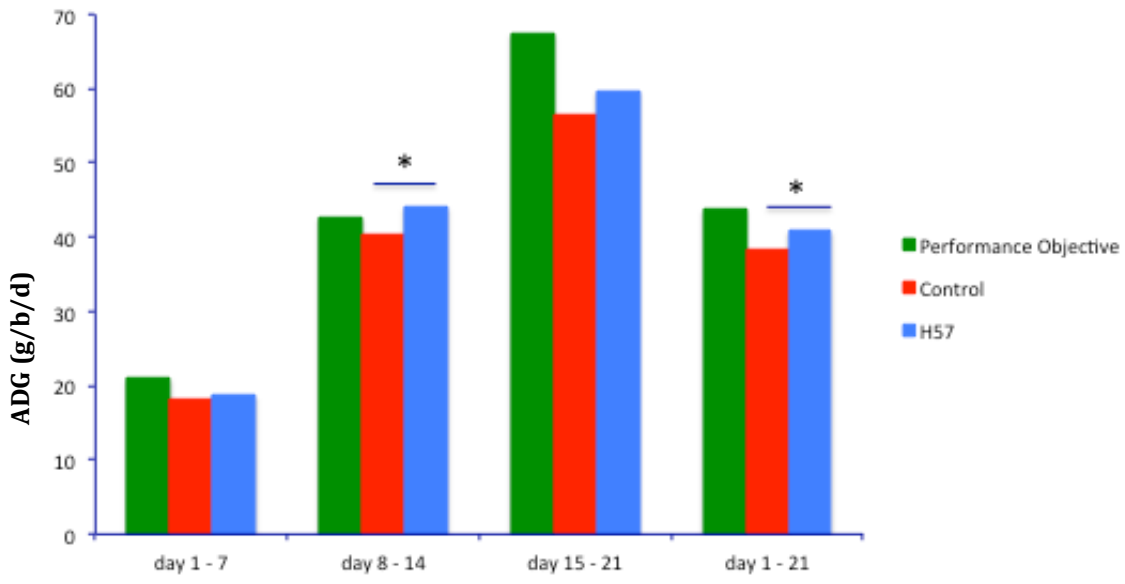


Figure 3-7: Average daily gain (g/bird/day) of control and H57 birds at different age in experiment 1 and comparison with performance objective (Aviagen, 2014b). *P<0.05

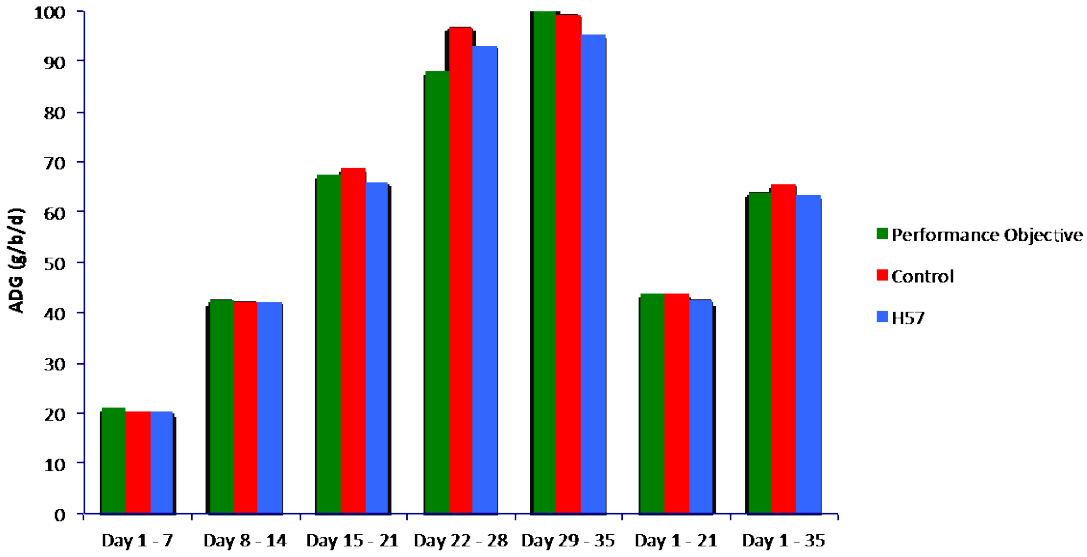
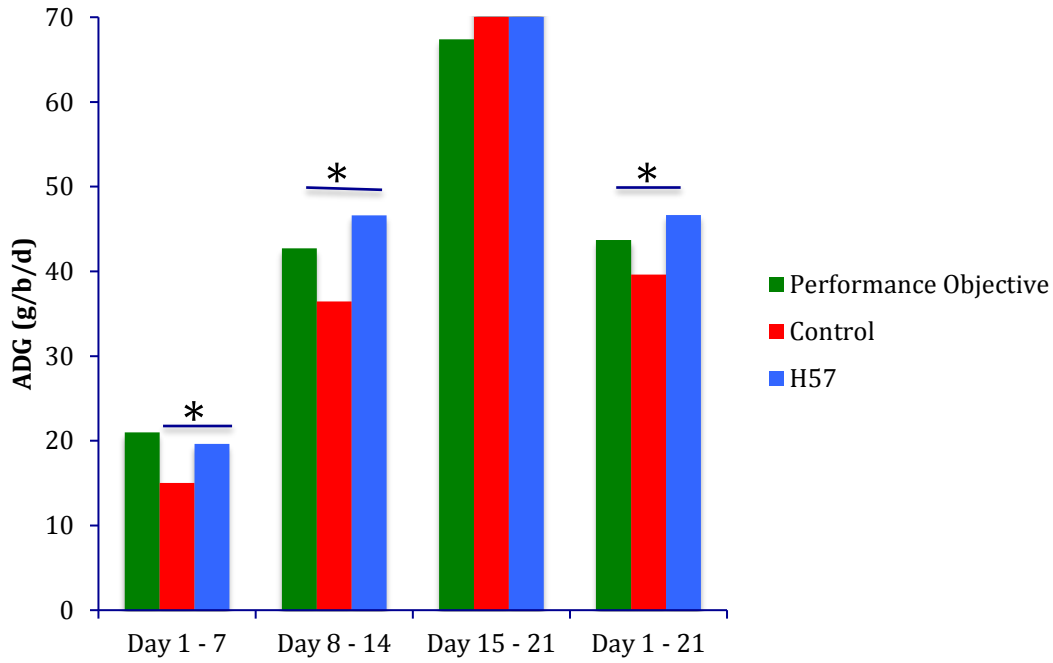


Figure 3-8: Average daily gain (g/bird/day) of control and H57 birds at different age in experiment 2 and performance objective (Aviagen, 2014b).

a)



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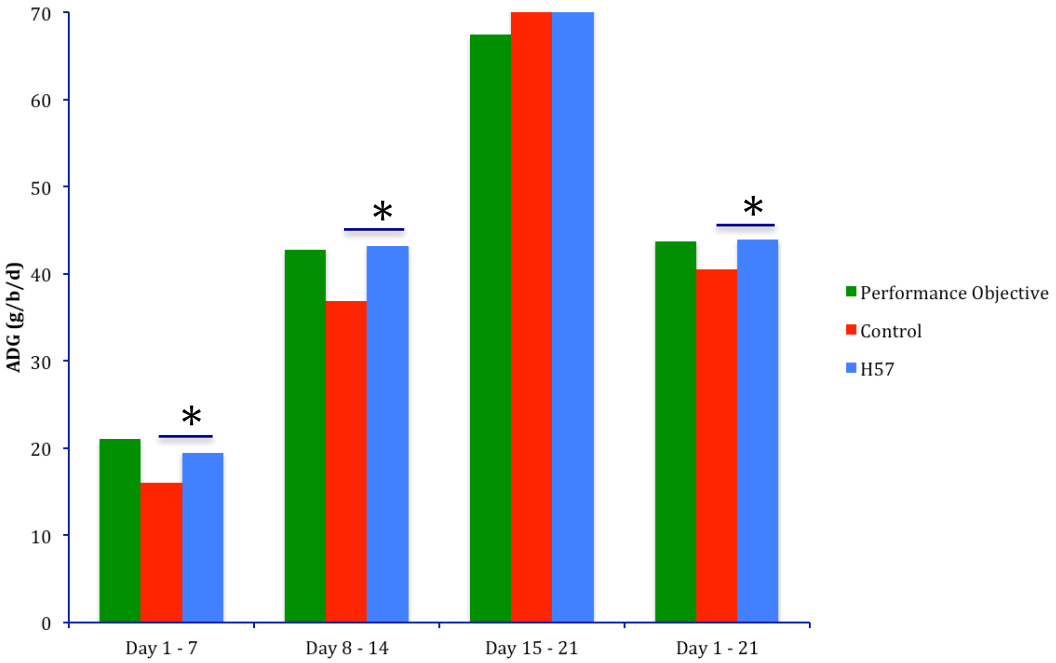


c)

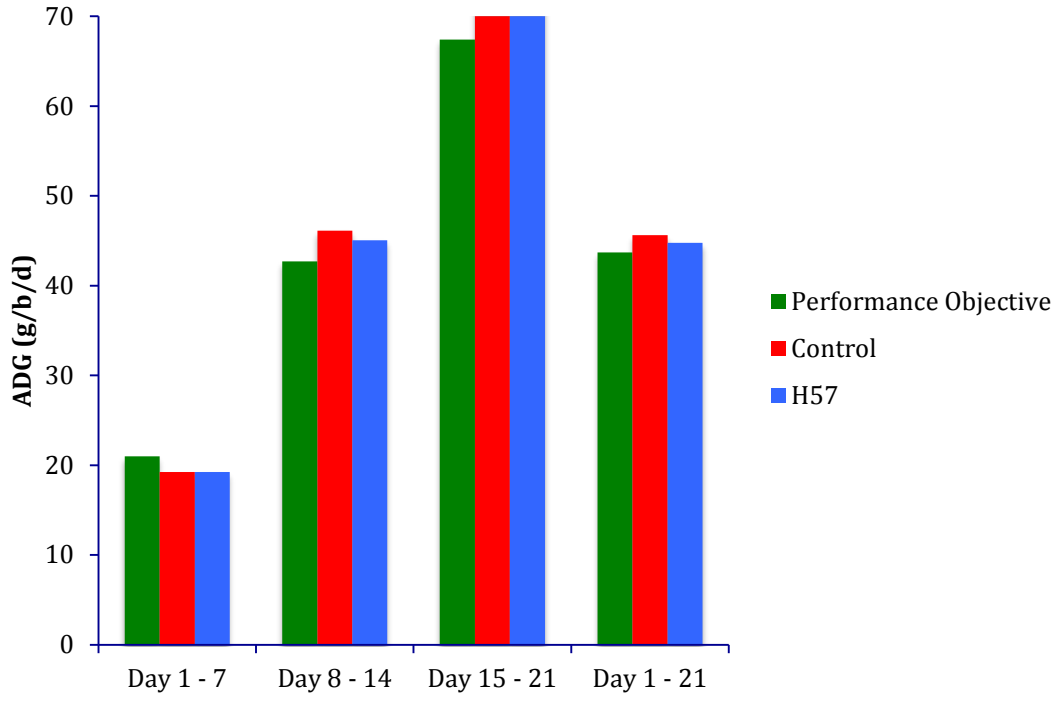


Figure 3-9: Average daily gain (g/bird/day) of control and H57 birds at different age in experiment 3 and comparison with performance objective (Aviagen, 2014b) a) sorghum based diet b) wheat based diet c) sorghum and wheat mixed diet.

Past studies by Ahmed *et al.* (2014), Lei *et al.* (2015) and An *et al.* (2008) have also demonstrated positive effects of feeding *B. amyloliquefaciens* on the growth rate of broiler chickens. Notably, in

all of these studies, growth rate of control (non-treated) birds had been supressed (Figure 3-10) as compared to a performance objective, in agreement with our experiments with H57.

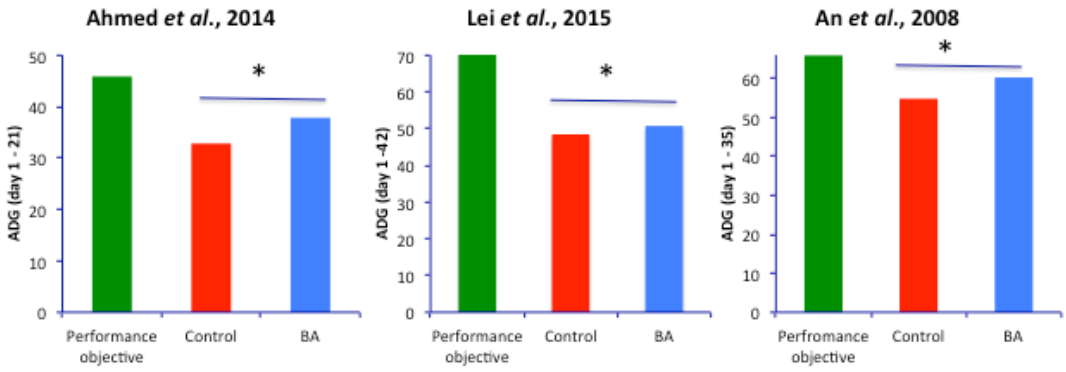


Figure 3-10: Growth rate of chickens and standard breed specific performance objectives (Aviagen, 2014b, Aviagen, 2014a) in control and *Bacillus amyloliquefaciens* treated (dietary administration) birds in past studies. ADG = average daily weight gain (g/bird/day). BA = Bacillus amyloliquefaciens. *p<0.05

In the experiments reported in this thesis, results appear to be related to diet composition and possibly the source of the chicks used in the experiments. Chickens fed either sorghum or wheat based diets without *B. amyloliquefaciens* H57 grew the slowest in our studies (experiment 1 and experiment 3). Sorghum contains anti-nutritional compounds such as kafirin as the major storage protein, phytate and/or tannin (Johns and Brewster, 1916; Bryden et al 2008). Steaming sorghum in pellet formation may induce an interaction between kafirin protein bodies and starch granules inhibiting the digestion of the starch. Kafirin and tannin seem to act together to reduce amino acid digestibility and total metabolisable energy of feed for poultry but it is not clear whether a similar interaction occurs with unheated mash feed. The amino acids in kafirin are poorly digested and deficient in essential amino acids lysine and cysteine. Sorghum may also contain condensed tannins in significant amounts (Butler et al., 1984) which complex with proteins including enzymes and thereby reduces digestion and uptake of amino acids by poultry (Bryden et al 2009). Because of these interactions sorghum is often regarded as an inferior grain for poultry feeds.

More than 80% of total phosphorus (P) in sorghum and more than 70% of total P in wheat (Selle et al., 2000) is present as phytate-bound P (phytate-P). Mono-gastric animals like poultry cannot efficiently use phytate-bound P in their diet (Woyengo and Nyachoti, 2013, Ravindran et al., 2000, Nelson, 1967). Moreover, phytates are able to complex protein thereby reducing the digestibility of nitrogen and essential amino acids by poultry (Ravindran et al., 2000, Selle et al., 2000, Woyengo and Nyachoti, 2013). Surprisingly H57 does not appear to produce phytase even though other *B. amyloliquefaciens* strains do (Le et al., 2016).

The anti-nutritional compounds in sorghum and/or wheat, for example non-starch polysaccharides or NSPs present in wheat, may reduce broiler growth rates. However, further study is needed to confirm whether this is the case in our study and how H57 assists in improving the feed value of ingredients by overcoming these effects.

Bacillus amyloliquefaciens produces a large range of extracellular metabolites eg. enzymes such as α-amylase, proteases, cellulase, xylanase etc. (Gracia et al., 2003, Lee et al., 2008, Gould et al., 1975, Breccia et al., 1998), antimicrobial and antifungal lipopeptides eg. surfactin, fengicin, bacillumycin D, iturin A (Koumoutsi et al., 2004, Ongena and Jacques, 2008, Chen et al., 2009, Arrebola et al., 2010), polyketides eg. macrolactin, difficidin, bacillaene, chlorotetain (Schneider et al., 2007, Chen et al., 2006, Rapp et al., 1988) and bacteriocin (Ulyanova et al., 2011). Strain H57 has genes to encode many of these exogenous metabolites including several carbohydrate activated enzymes including glycoside hydrolases, lipopeptides (surfactin, iturin, bacillomycin D and fengycin) and antibiotic polyketides (macrolactin, difficidin and bacillaene) (Schofield et al., 2016). At this stage, it is unknown whether these compounds are of benefit in poultry nutrition.

Bacillus amyloliquefaciens has also been used to improve the quality of non-conventional feedstuffs, by solid-state fermentation, for use as potential ingredients in poultry diets (Wizna et al., 2009, Wizna et al., 2008). The solid-state fermentation by *B. amyloliquefaciens* increased crude protein and decreased crude fibre of non-conventional feedstuffs (tapioca by-products, sago pith and rumen content) (Wizna et al., 2008, Wizna et al., 2009). Recently, Chistyakov et. al (2015) used solid-state *B. amyloliquefaciens* fermented soybean with retained spores and vegetative cells of *B. amyloliquefaciens* to inoculate a poultry diet, increasing body weight by 7 to 8% and improving FCR by up to 9%.

There appear to be a number of ways in which H57 could influence bird performance and one could well be the modification of the gastrointestinal microbiome. In recent times, there has been a surge in interest regarding the role of the gastrointestinal microbiota in shaping the health and energy balance of the host. Study of the effects of H57 on intestinal microbiota could provide further insights on the mode of action of this probiotic.

Chapter 4 Microbial community structure in the gastrointestinal tract of broiler chickens fed Bacillus amyloliquefaciens H57

4.1 Introduction

The gastrointestinal tract (GIT) of the chicken is a complex microbial with dynamic consortium of microorganisms, both in the lumen and on the mucosal surface with commensal (or probably symbiotic) relationship among themselves and with the host (Yeoman et al., 2012, Saengkerdsub et al., 2007a, Saengkerdsub et al., 2007b, Apajalahti et al., 2004). Contribution of the gastrointestinal microbiota to animal health, overall wellbeing and productivity is well appreciated. The chicken gastrointestinal microbiome has been studied for more than four decades to understand its characteristics and function. The population and composition of this ecosystem are contingent mainly on the host diet (Apajalahti et al., 2001), and to a lesser extent on systemic immune response, GIT secretions and on the type of litter material used (Torok et al., 2009). However, composition of the ileal and caecal microbiome can significantly differ (Mohd Shaufi et al., 2015). Composition, physical state and nutrient concentration of the animal diet and presence of feed additives have significant effects on the intestinal microbial dynamics (Thompson et al., 2008, Knarreborg et al., 2002b, Singh et al., 2013, Engberg et al., 2000, Engberg et al., 2004) which in turn affect the bird health, performance and physiological state (Round and Mazmanian, 2009, Nicholson et al., 2005). Here, we extend these findings by characterising the effects of H57 on the ileal and caecal microbiota of broiler chickens using high-throughput culture independent DNA sequencing (Illumina).

Probiotics can change the microbial population dynamics in the GIT (Mountzouris et al., 2007, Mountzouris et al., 2009, An et al., 2008). Earlier studies on the effects of probiotics on the GIT microbial population of poultry using traditional microbial growth methods were unable to adequately unravel the complexity of gastrointestinal microbial ecosystem. However, recent developments in culture independent techniques through the use of molecular biology tools and bioinformatics have enabled an in-depth study with an increasing database and understanding (van der Hoeven-Hangoor et al., 2013, Thompson et al., 2008, Torok et al., 2009, Cressman et al., 2010, Pissavin et al., 2012, Ammor et al., 2008). This study used next generation sequencing of DNA to study the microbiome composition of the ileum, caeca and faeces of broiler chickens. This study addresses the question “can the gastrointestinal microbial ecology be modified to improve production efficiency in chicken using feed additives such as probiotics?”

The 16S rRNA gene is one of the most widely used molecular markers in the study of microbial community structure. All bacterial species carry at least one copy of this gene with highly conserved and variable nucleotide sequences, which make the comparison between distantly related microbes and assessment of similarity between closely related organisms (more details in chapter 2) (Olsen et al., 1986, Case et al., 2007). The 16S rRNA amplicon sequencing by using next generation DNA sequencing technique was used to study the effects of H57 on the microbial population of the ileum and caecum.

4.2 Materials and methods

4.2.1 Experimental design

Details about experimental design have been presented in the previous chapter (chapter 3). Briefly, experiment 1 was undertaken with a sorghum based diet consisting of two treatments (basal diet ± H57) and 6 replicates per treatment. Experiment 2 was carried out with a wheat based diet with and without H57 consisting of 12 replicates per treatment (half in cages and half in floor pens). Similarly, experiment 3 consisted of three types of diet (wheat, sorghum and wheat and sorghum mix) with and without H57 each with 6 replicates. Detail composition of each diets is given in chapter 3.

4.2.2 Sample collection

Samples of intestinal content from the ileum and caeca were collected on day 21 from experiment 1, on day 35 from experiment 2 and on day 4, day 13 and day 21 from experiment 3. Samples were collected from 24 birds (two birds from each replicate) in experiment 1, 24 birds (two birds from 50% of replicates selected randomly) in experiment 2 and 36 birds (one bird from each replicate) at each time point (day 4, day 13 and day 21) in experiment 3. Approximately 0.5 g of digesta from the ileum and caeca were collected into 1.5 ml Eppendorf tubes by squeezing the content directly into the tubes from the intestine to prevent contamination. Samples from the ileum were collected from the distal part of the ileum while samples from the caeca were collected after removing about 2 cm from the proximal end. Faecal samples were also collected at day 13 from each cage from experiment 3. Pieces of paper were kept under each cage and about 0.5 g of faecal samples were collected and placed into 2 ml Eppendorf tubes. The samples were immediately frozen in liquid nitrogen, kept on dry ice during collection and transportation, and stored at -80°Ç. Details of samples collected for microbial profiling are given in Table 4-1.

Table 4-1 Details of samples used for 16S rRNA gene amplicon sequencing

Experiment No	Diet type	Age (day)	Site and no. of samples		
			Ileum	Caecum	Faeces
1	Sorghum based	21	24	24	-
2	Wheat based	35	24	24	-
3	Sorghum based	4	12	12	-
	Wheat based		12	12	-
	Sorghum+wheat mix		12	12	
	Sorghum based	13	12	12	12
	Wheat based		12	12	12
	Sorghum+wheat mix		12	12	12
	Sorghum based	21	12	12	
	Wheat based		12	12	
	Sorghum+wheat mix		12	12	

4.2.3 DNA extraction

DNA from digesta samples was extracted by a modified repeated bead beating plus column (RBB+C) method (Yu and Morrison, 2004) and the QIAamp Fast DNA Stool Mini Kit (QIAGEN, Velno, The Netherlands). Briefly, 0.2 g of digesta samples were weighed into sterile bead beating tubes containing 0.5 g of 0.1 mm zirconia beads (BioSpec Products Inc., Oklahoma, USA) and suspended in 1 ml of lysis buffer (2.9% NaCl, 0.6% Tris, 0.05M EDTA pH 8.0 and 4% SDS). The suspension was homogenized twice in a mini bead beater (BioSpec Products Inc, Oklahoma, USA) for 5 minutes each, then heated at 70°C for 5 minutes followed by centrifugation (Eppendorf 5424) at 20,000 g for 3 minutes to separate bacterial genomic DNA from the digesta. The separated supernatant was then treated with 1 ml of InhibitEX buffer from the kit to neutralize any PCR inhibitors present in the digesta samples followed by centrifugation at 20,000 g for 6 minutes to separate DNA from any debris present in the samples and incubated at 37°C for one hour with 20 µl (40 mg/ml) of DNase free RNase for ileal samples or 30 µl (40 mg/ml) of DNase free RNase for caecal samples. The samples were then transferred into 15 ml Falcon tubes containing 25 µl of Proteinase K (QIAGEN, Velno, The Netherlands), added 600 µl of buffer AL, vortex mixed and heated at 70°C for 10 minutes. Absolute ethanol (1.3 ml) was added in the tube and all the liquid in the tube was spun down through a QIAamp spin column by adding 600 µl at a time. DNA in the column was washed with 500 µl of AW1 (wash buffer 1) and AW2 (wash buffer 2) according to the manufacturer's directions and finally eluted with either 100 µl (ileum) or 200 µl (caecum) of elution

buffer (10mM Tris-cl, pH 8.5). The extracted genomic DNA was stored at -20°C until further analysis.

4.2.4 Preparation of 16S rRNA gene amplicon libraries and sequencing

To prepare for the sequencing the concentration of DNA in the genomic DNA samples was measured using a Qubit 2.0 fluorometer (Thermo Fisher Scientific Inc, Victoria, Australia) and diluted with ultrapure distilled water to 5 ng/ μ l.

Four samples (1 each from Control and H57 from both sites – ileum and caecum) were selected to be sequenced initially twice by using two sets of primers, 926F/1392R and 803F/1392R for the amplification (Table 4-2). The primer pair 926F/1392R are specific to the V6-V8 variable region of the 16S rRNA gene of bacteria and archaea and 18S rRNA gene of eukarya (Engelbrektson et al., 2010). Similarly, the primer pair 803F/1392R targets the V8 region of the 16S rRNA gene of bacteria and archaea but not the eukaryotic 18S rRNA gene (Goodfellow and Stackebrandt, 1991). Both primer pairs gave similar results. Universal primer pair 926F and 1392R were subsequently chosen for the amplification of DNA to be sequenced.

Table 4-2: Primers used for the amplification of DNAs to be sequenced

Primers	Sequence (5' - 3')
803F	ATTAGATACCCTGGTAGTC
926F	AAA CTY AAA KGA ATT GAC GG
1392R	ACG GGC GGT GTG TRC

The 16S rRNA gene sequencing library was prepared by standard library preparation method for Illumina, following the manufacturer's protocol (Illumina Inc., San Diego, CA, USA). Two rounds of PCR reactions were undertaken to prepare the sequencing library. In the first round, the V6 -V8 hypervariable region of 16S rRNA gene was amplified (primer pair 926F/1392R (0.2 μM each)) and included a 'tag' sequence complementary to the Illumina sequence adapter, in 25 μ l reactions with 1x KAPA HiFi HotStart ReadyMix (Kapa Biosystems Inc., Wilmington, MA, USA). The PCR reaction parameters comprised an initial denaturation at 95°C for 3 min followed by 25 cycles of 95°C for 30 s for denaturation, 55°C for 30 s for annealing and 72°C for 30 s for elongation, with a final extension for 5 min at 72°C. In the second round, indexing PCR was carried out in 50 μ l reactions with Illumina sequencing adapters and dual indexing barcodes using 5 μ L of template DNA, 5 μ L of each index primer and 25 μ l of 2x KAPA HiFi HotStart ReadyMix. The PCR reaction parameters comprised an initial denaturation at 95°C for 3 min followed by eight cycles of

95°C for 30 s for denaturation, 55°C for 30 s for annealing and 72°C for 30 s for elongation, with a final extension for 5 min at 72°C. The resulting amplicon libraries were cleaned using Agencourt AMPure XP beads (Beckman Coulter Australia Pty Ltd, Lane Cove, NSW, Australia) following manufacturer's standard protocols and sequenced using Illumina NextSeq platform (Illumina, San Diego, CA, USA).

4.2.5 Quality control and sequence processing

Quality of the sequences was checked with fastQC (Andrews, 2010). The primer sequences were removed by trimming the first 20 bases. Quality filtering was done by Sliding Window quality filtering approach of Trimmomatic software truncating 3′ end of sequences at the first residue with an average base quality below 15 (Bolger et al., 2014). All sequences were finally trimmed to 250 bases and translated to fasta format while reads with less than 250 bases were rejected. The sequences were then clustered into operational taxonomic units (OTUs) at 97% DNA sequence similarity using Quantitative Insights Into Microbial Ecology (QIIME) software (Caporaso et al., 2010). Any OTU having less than 0.05% abundance were not included in subsequent analysis. OTUs were then identified by using Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990) against Greengenes database version May 2013 and an OTU table with relative abundance for each OTU was generated. OTU numbers were adjusted for 16S gene copy number variances using software CopyRighter (Angly et al., 2014).

4.2.6 Statistical analysis

Further data analysis was done using R programming language (R Core Team, 2016) in RStudio, an integrated development environment for R. Significance of difference in microbial population between treatments (Control and H57) was tested by Permutational Multivariate Analysis of Variance (PERMANOVA) of operational taxonomic units (OTUs) using adonis() function of the vegan package (Oksanen et al., 2016). Ordination of the samples (individual chickens) was done by principal component analysis (PCA) of Hellinger transformed (Legendre and Gallagher, 2001) relative abundance of OTUs data using vegan package in R (Oksanen et al., 2016). Monotonic relationships between relative abundance of OTUs and body weight of individual sampled chickens was analysed by using Spearman's correlation test with ggscatter() function of the package ggpubr after testing the normality of the distribution of the data with the shapiro.test() function. Comparison of relative abundance of individual taxons (OTU to phylum) was done by T-Test (two-tailed two-sample unequal variance).

4.3 Results

4.3.1 Effects on intestinal microbiota of chicken fed sorghum based diet (experiment 1)

PERMANOVA of operational taxonomic units from Control and H57 treated birds indicated that H57 significantly modified the microbial community structure both in the ileum ($P = 0.001$) and caecum ($P = 0.001$) with significant change in the relative abundance of multiple bacterial taxa (Table 4-3, Table 4-4, Figure 4-1, Figure 4-2, and Figure 4-3). Microbiota diversity (Shannon index) was significantly reduced ($P = 0.023$) in the ileum by dietary H57 addition (Control 4.35 vs H57 group 3.92) while diversity was not affected in the caecum (rarefaction with sequencing depth of 25,000 reads per sample in both sites). Among three experiments, H57 had the most prominent effects on the intestinal microbial profile in experiment 1. Therefore, results about the effects of H57 on the microbial profile of ileum and caecum in experiment 1 have been presented in detail.

Streptococcus and Lactobacillus were the dominant OTUs in the ileum both in Control and H57 treated birds while Faecalibacterium were dominant in the Controls and Bacteroides were dominant in the H57 treatment group in the caecum. Relative abundance of the genera Streptococcus and Bacillus increased ($P < 0.05$) while that of genera Staphylococcus, SMB53 and Blautia and families Peptostreptococcaceae and Clostridiaceae were reduced ($p < 0.05$) for the birds given H57. In the caecum, relative abundance of the genus Bacteroides and Bacillus increased while the relative abundance of the genera Staphylococcus, Lactococcus, Faecalibacterium, Coprobacillus and Adlercreutzia and family Clostridiaceae decreased. The most prominent change was an increase in the relative abundance of Bacteroides in the caecum from 0.0002% relative abundance in Control birds to 17.4% in the H57 treated birds; becoming the most dominant taxon in the caeca. Major OTUs (>1% relative abundance) with significantly different relative abundance between Control and H57 are shown in Table 4-3 and Table 4-4.

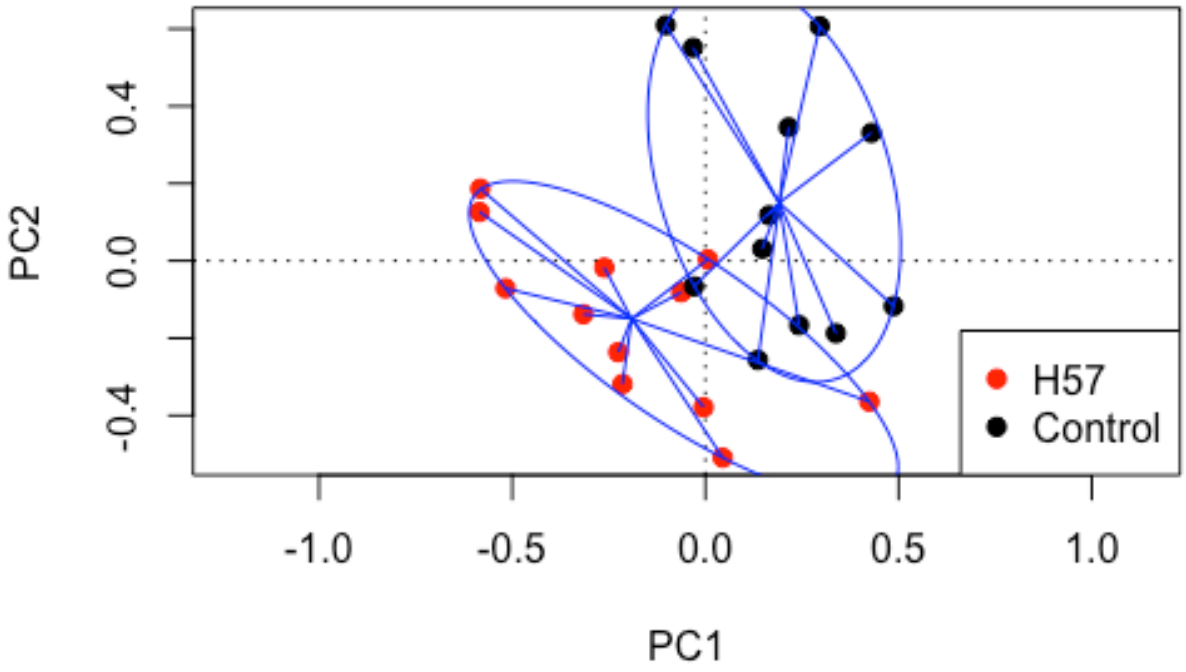


Figure 4-1 Ordination of samples (individual chickens) by Principle Component Analysis (PCA) of normalised relative abundance of OTUs from ileum (experiment 1).

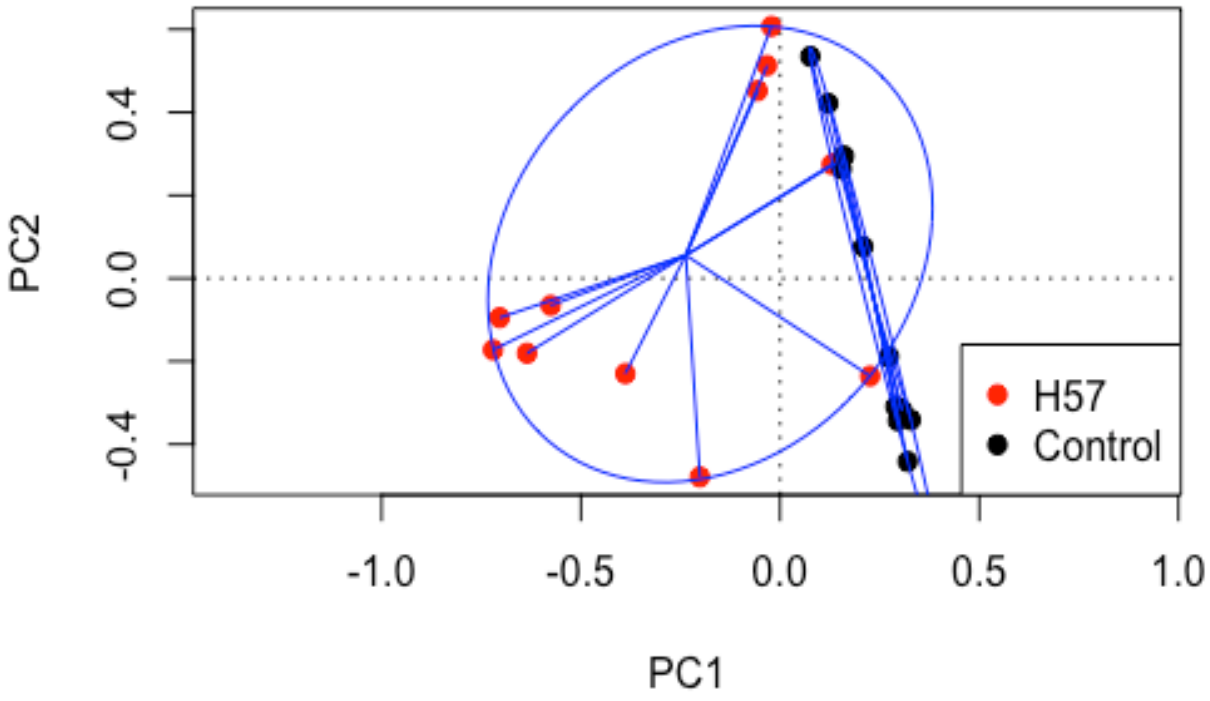


Figure 4-2 Ordination of samples (individual chickens) by Principle Component Analysis (PCA) of normalised relative abundance of OTUs from caecum (experiment 1).

Table 4-3 Average relative abundances of OTUs (with more than 1% relative abundance) significantly altered due to H57 in ileum of birds on sorghum based diets (experiment 1). k = kingdom, p = phylum, c = class, o = order, f = family, g = genus, s = species

OTUs	Average relative abundance		
	Ileum Control	Ileum H57	P-Value
k__Bacteria; p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Streptococcaceae; g__Streptococcus; s__alactolyticus	16.61	28.82	<0.001
k__Bacteria; p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Lactobacillaceae; g__Lactobacillus; s__	1.52	5.78	0.001
k__Bacteria; p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Lactobacillaceae; g__Lactobacillus; s__salivarius	3.08	5.12	0.015
k__Bacteria; p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Lactobacillaceae; g__Lactobacillus; s__salivarius	3.14	4.80	0.022
k__Bacteria; p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Enterococcaceae; g__Enterococcus; s__	2.17	1.94	<0.001
k__Bacteria; p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Streptococcaceae; g__Streptococcus; s__alactolyticus	1.83	1.36	<0.001
k__Bacteria; p__Firmicutes; c__Bacilli; o__Turicibacterales; f__Turicibacteraceae; g__Turicibacter; s__	4.38	1.35	0.026
k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Enterobacteriales; f__Enterobacteriaceae; g__; s__	0.07	1.34	0.001
k__Bacteria; p__Actinobacteria; c__Actinobacteria; o__Actinomycetales; f__Corynebacteriaceae; g__Corynebacterium; s__stationis	1.67	1.23	0.011
k__Bacteria; p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Streptococcaceae; g__Streptococcus; s__alactolyticus	0.44	1.04	<0.001
k__Bacteria; p__Firmicutes; c__Bacilli; o__Lactobacillales; f__; g__; s__	0.45	1.03	<0.001
k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Clostridiaceae; g__SMB53; s__	1.23	0.81	0.002

At higher taxonomic level, there was no difference in phylum between Control and H57 group and significantly higher abundance of bacilli and lower abundance of clostridia (class) in H57 group compared to Control in the ileum (Figure 4-5). Similarly, order Lactobacillales (Figure 4-7) and families Streptococcaceae and Bacillaceae were significantly higher in H57 group (Figure 4-9) while order Clostridials (Figure 4-7) and families Preptostreptococcaceae, Clostridiaceae, Turibacteriaceae and Staphylococcaceae were significantly lower in H57 group as compared to Control (Figure 4-9).

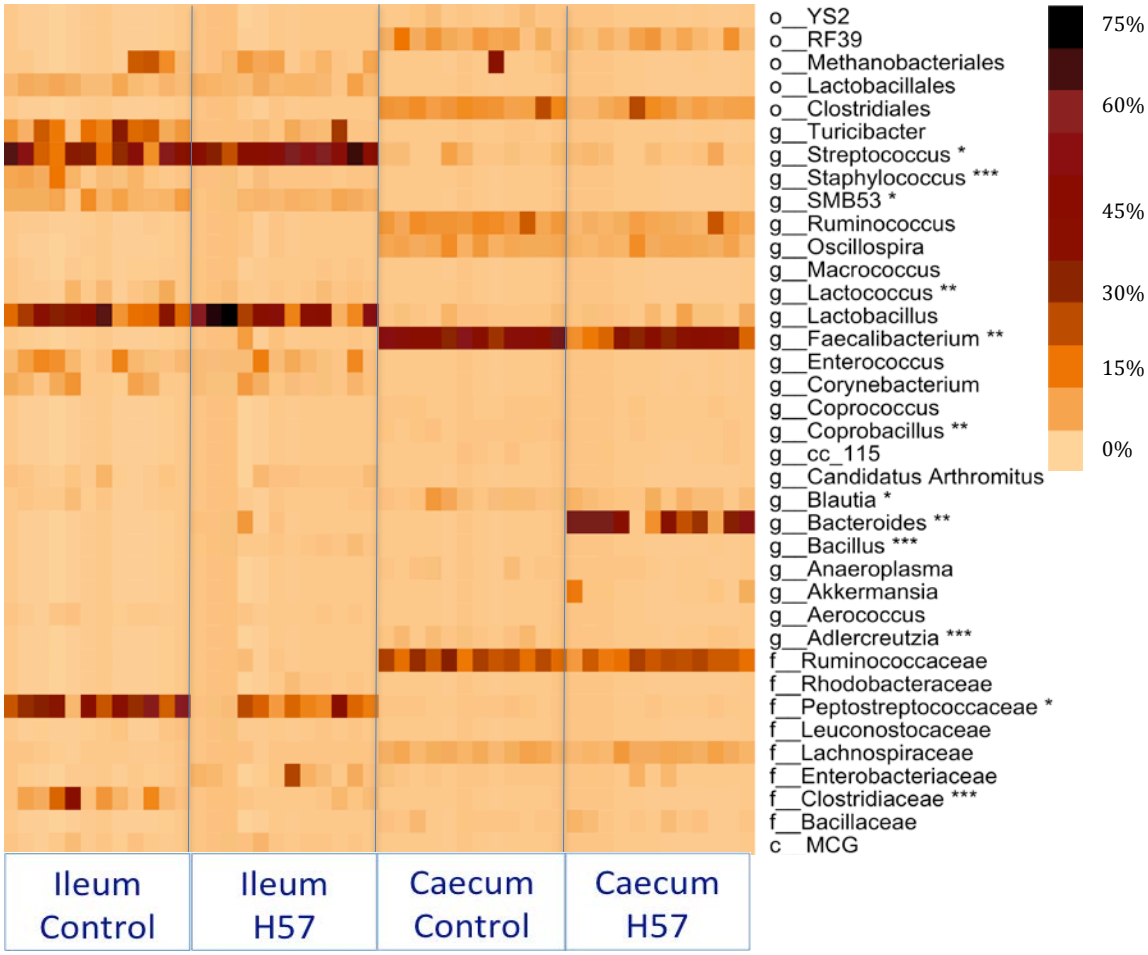


Figure 4-3 Heat map showing the relative abundance of bacterial taxa in ileum and caecum of control and H57 fed birds in experiment 1. Each column represents an individual bird and each row represents a bacterial taxon. *significant difference in ileum, **significant difference in caecum, ***significant difference in both ileum and caecum at $p < 0.05$.

Table 4-4 Average relative abundances of OTUs (with more than 1% relative abundance) significantly altered due to H57 in caeca of birds on sorghum based diets (experiment 1)

OTUs	Average relative abundance		
	Caecum Control	Caecum H57	P-Value
k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Ruminococcaceae; g__Faecalibacterium; s__prausnitzii	9.95	4.13	0.008
k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Bacteroidaceae; g__Bacteroides; s__	0.00	13.60	0.010
k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Bacteroidaceae; g__Bacteroides; s__fragilis	0.00	1.18	0.033
k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Bacteroidaceae; g__Bacteroides; s__fragilis	0.00	2.64	0.033

In caecum, family Firmicutes and Actinobacteria were significantly reduced in H57 group while Bacteroidetes were significantly increased (Figure 4-4). Class Clostridia had significantly lower relative abundance in H57 group but Bacteroidia had significantly higher abundance as compared to

Control (Figure 4-6). Similarly, order Clostridials (Figure 4-8) and family Ruminococcaceae (Figure 4-10) has been reduced and order Bacteroidels (Figure 4-8) and family Bacteroideceae has been increased in H57 group as compared to Control group (Figure 4-10).

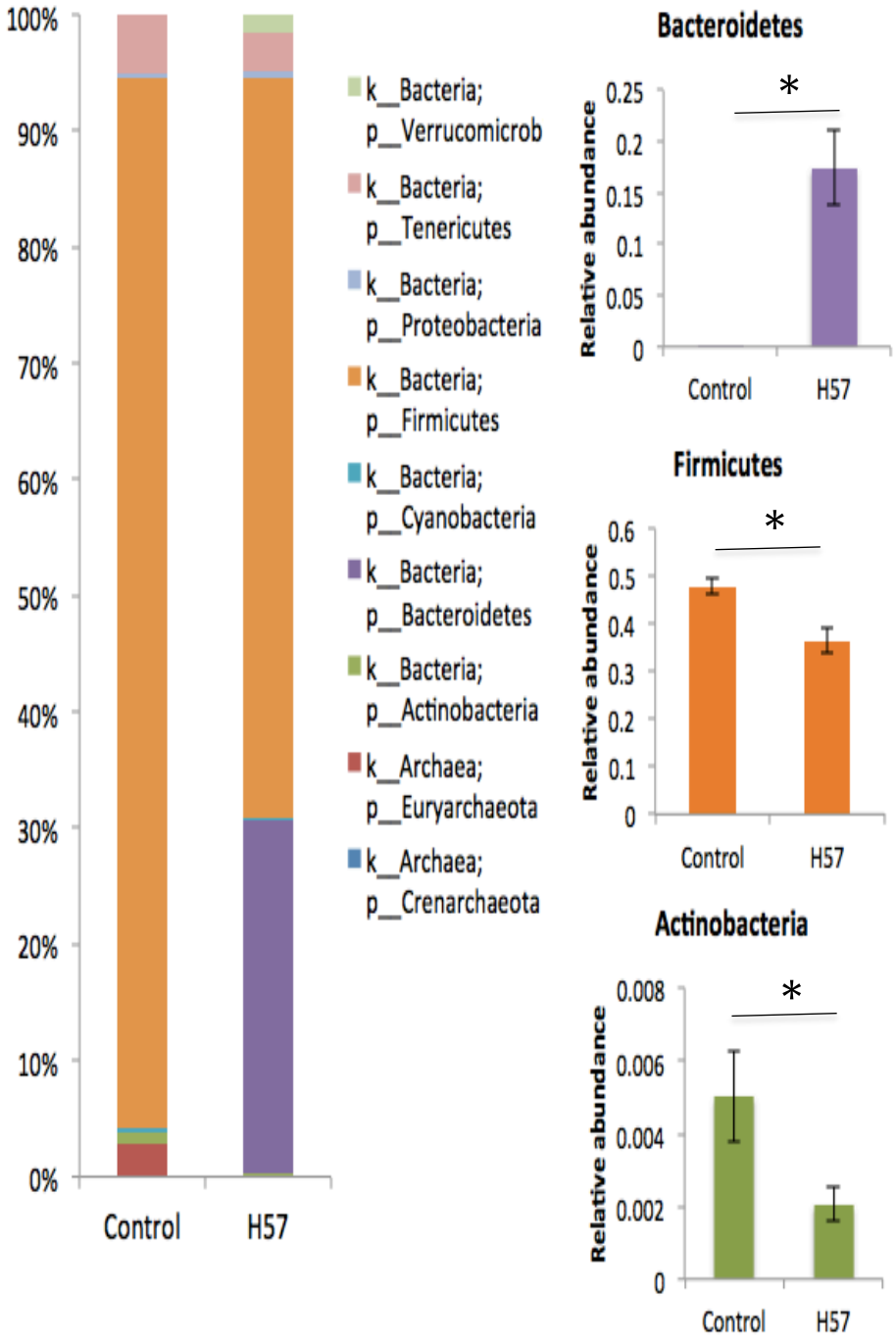


Figure 4-4 Relative abundance of different phyla in caecum in experiment 1. * (P<0.05). Error bars are standard error of mean (SEM)

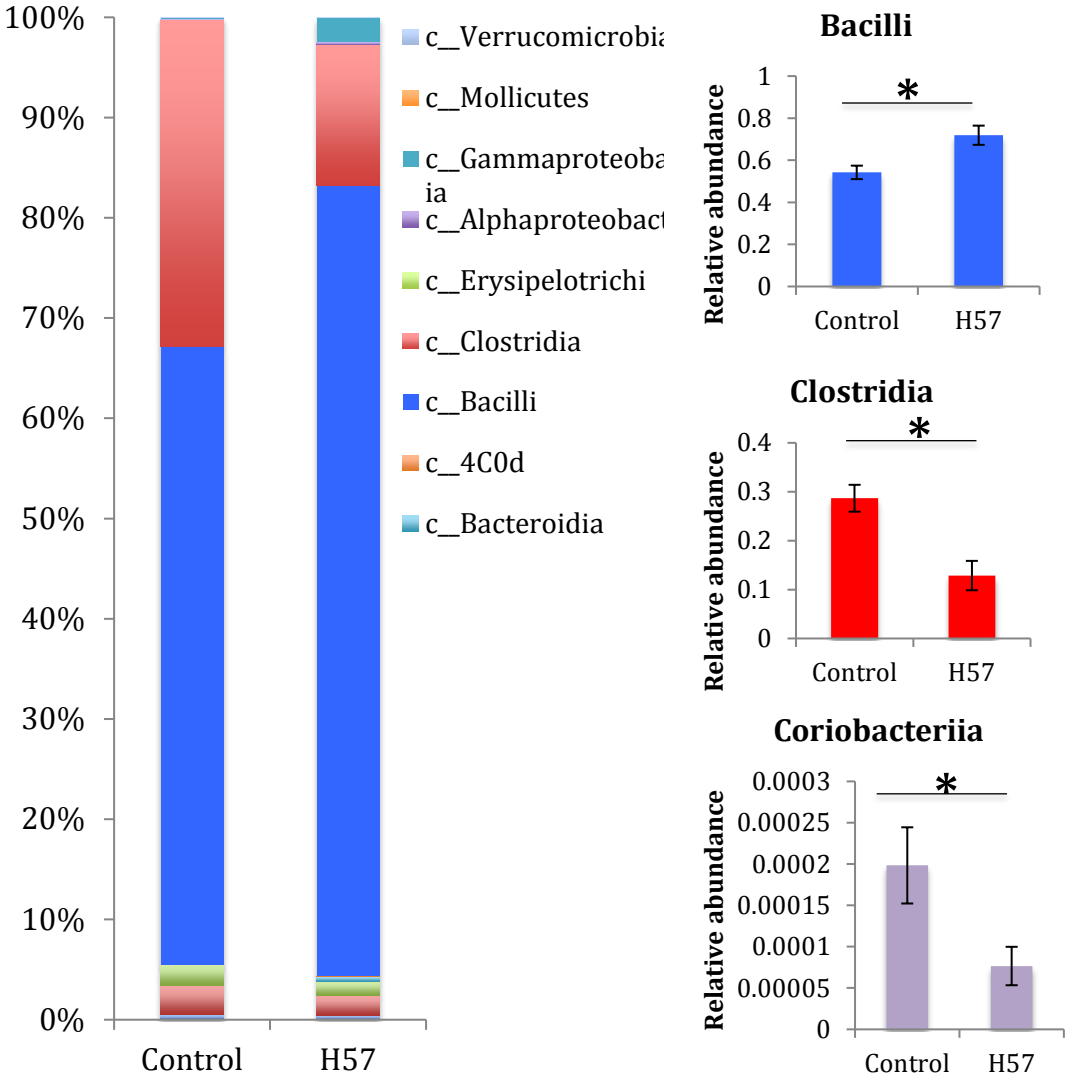


Figure 4-5 Relative abundance of different classes in ileum in experiment 1. * (P<0.05). Error bars are standard error of mean (SEM)

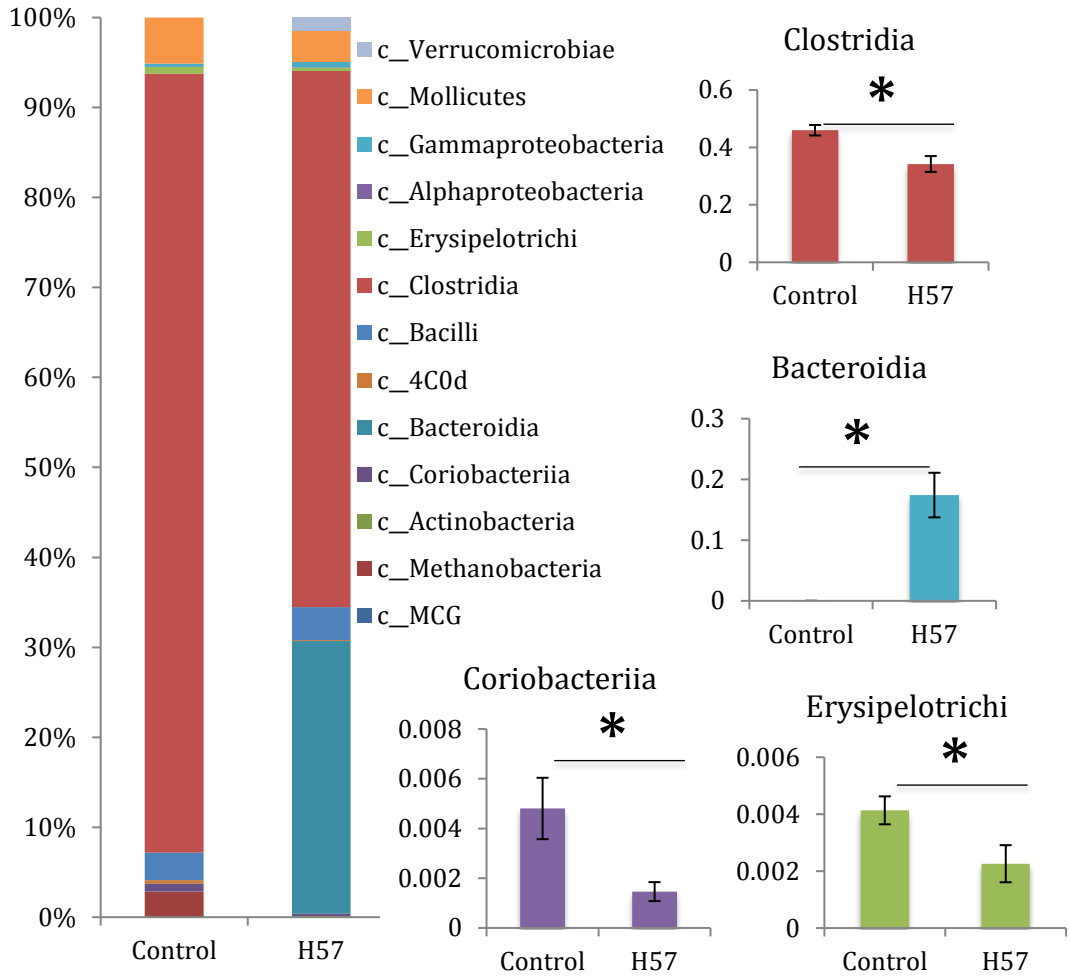


Figure 4-6 Relative abundance of different classes in caecum in experiment 1. * (P<0.05). Error bars are standard error of mean (SEM)

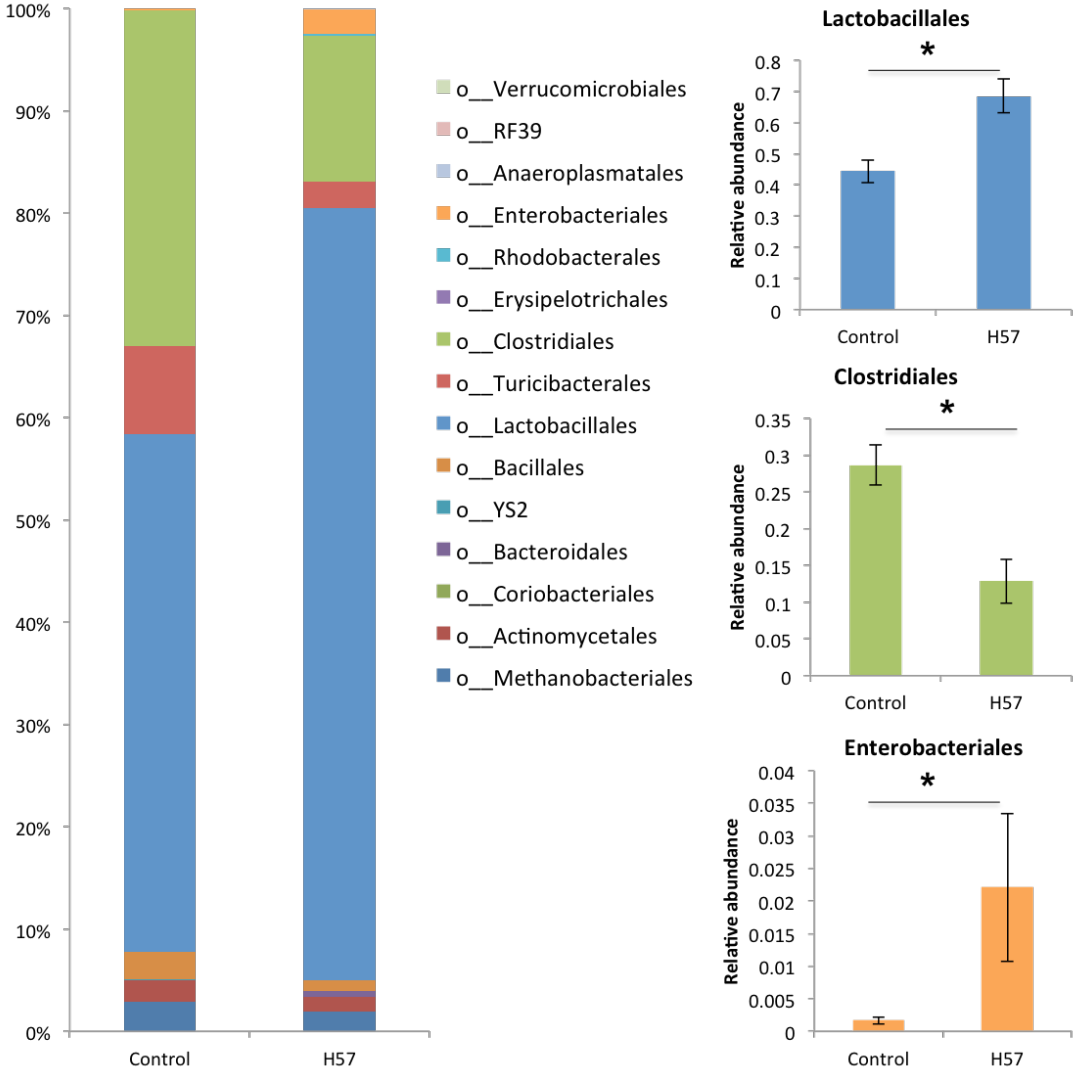


Figure 4-7 Relative abundance of different orders in ileum in experiment 1. * (P<0.05). Error bars are standard error of mean (SEM)

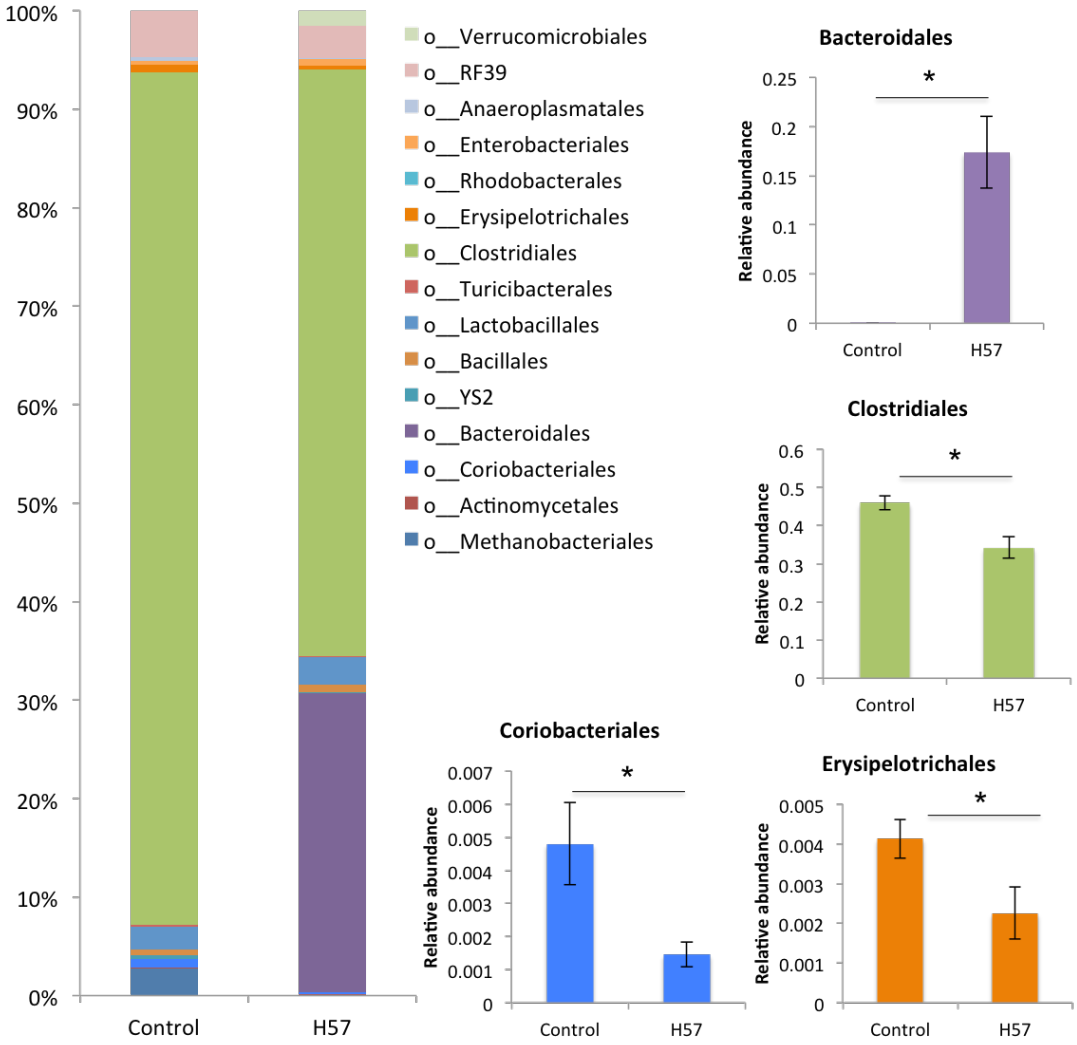


Figure 4-8 Relative abundance of different orders in caecum in experiment 1. * (P<0.05). Error bars are standard error of mean (SEM)

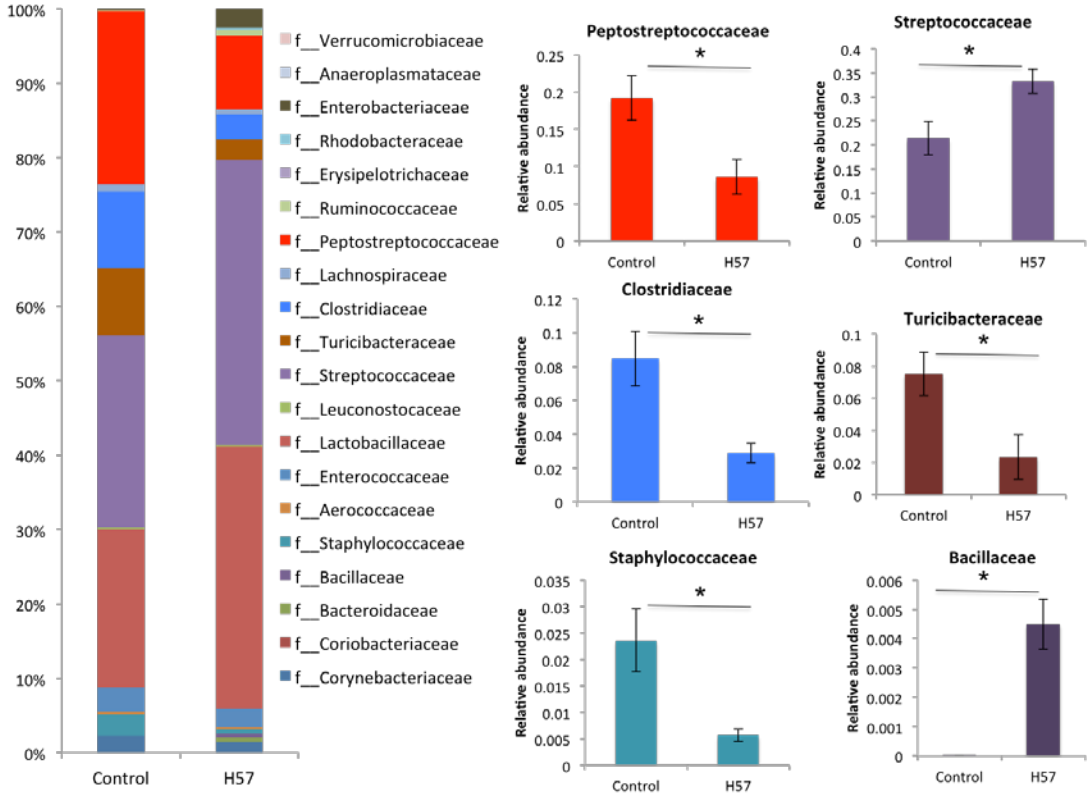


Figure 4-9 Relative abundance of different families in ileum in experiment 1. * (P<0.05). Error bars are standard error of mean (SEM)

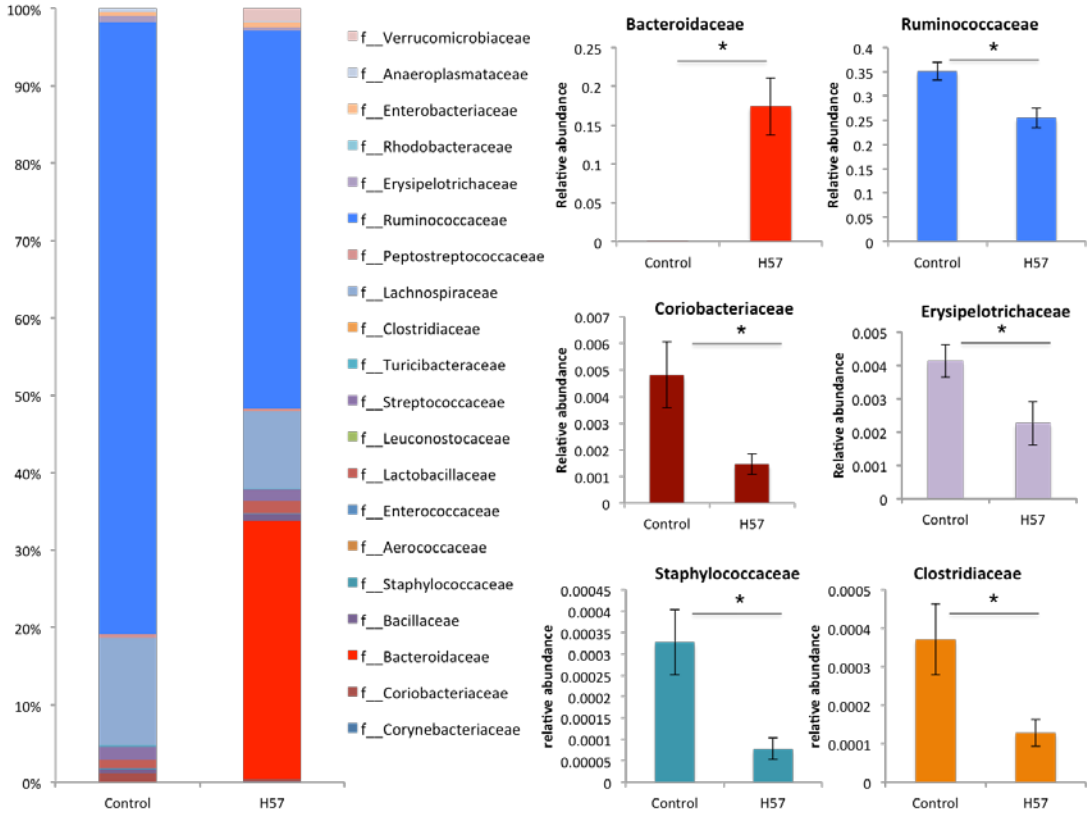


Figure 4-10 Relative abundance of different families in caecum in experiment 1. * (P<0.05). Error bars are standard error of mean (SEM)

4.3.2 Effects of H57 on intestinal microbial profile of chicken fed a wheat based diet (experiment 2)

When chickens were raised on a wheat based diet for 35 days, H57 had a significant effect on the microbial community profile of the caecum (Figure 4-11, P = 0.04, PERMANOVA) but not of the ileum (P = 0.37, PERMANOVA).

In the ileum, none of the major OTUs except two minor OTUs (order Clostridiales and genus *Bacillus*) had significantly different average relative abundance between Control and H57 treated chickens.

In the caecum, five OTUs had significantly different average relative abundance between Control and H57 groups. Two of these OTUs (families Rikenellaceae (0.005% in Control vs 3.8% in H57) and Lachnospiracea (2.1% in Control vs 0.9% in J57)) had relative abundance of greater than 1% at least in one treatment group (Control or H57).

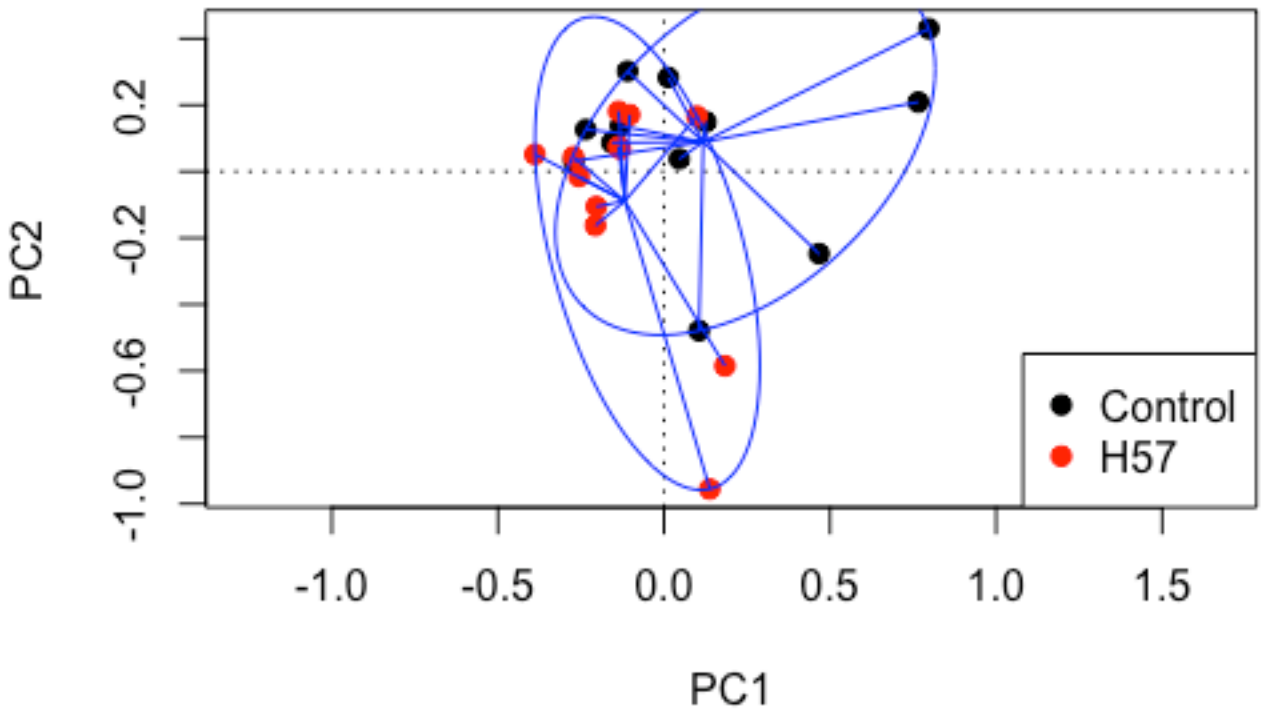


Figure 4-11 Principle Component Analysis (PCA) of normalized relative abundance of OTUs from caecal digesta samples at day 35 (experiment 2).

4.3.3 Intestinal microbiota of chickens raised in wire cages and floor pens (deep litter) were not different

In the first experiment the birds were housed on the floor with a simulated deep litter. As it was planned to conduct future experiments with cages in a temperature controlled room, we compared bird performance raised in the litter system with those in metal cages, both receiving the same diet, in this case a wheat based diet, with and without H57. We analysed the ileal and caecal microbiota of birds receiving Control diet (without H57) both in pens and cages to examine the effect of preventing coprophagy in cages on intestinal microbiota.

PERMANOVA of microbial community data indicated that there was no significant difference in microbial community structure both in the ileum ($P > 0.05$) and caecum ($P > 0.05$) associated with housing type.

The dominant OTUs in the ileum were *Lactobacillus* and the dominant OTUs in caecum were *Faecalibacterium* both in the cages and deep litter pens. However, the relative abundance of two of the OTUs representing *Lactobacillus* were significantly lower ($P < 0.05$, 1st 0.7% in pens vs 0.1% in cages, 2nd 2.1% in pens vs 0.3% cages) in the ileum of the birds raised in cages than in the birds kept in deep litter pens. Relative abundance of one OTU representing *Ruminococcus* was significantly increased ($P < 0.05$, 0.1% in pens vs 0.6% in cages) in the caecum of birds in cages compared to those in the deep litter pens. Microbial diversity (Shannon index) was similar ($P > 0.05$) in both housing systems (rarefaction with sequencing depth of 4000 reads per sample from both sites).

4.3.4 Effects on intestinal microbiota of chickens fed sorghum, wheat and wheat plus sorghum blend diets (experiment 3)

4.3.4.1 Sorghum based diet

Analysis of normalized relative abundances of OTUs showed that overall microbial population structures of Control and H57 treated chickens fed a sorghum based diet were significantly different on day 4 in the caecum (Figure 4-12, $P = 0.027$) and on day 13 in the ileum (Figure 4-13, $P = 0.024$). There were no significant differences in the overall structure of the microbial populations between Control and H57 in the ileum on day 4, caeca on day 13 and both the ileum and caeca on day 21. Dominant OTUs in the ileum were genus *Enterococcus* both in the Control and H57 on day 4, genus *Lactobacillus* in Control and genus *Candidatus Arthromitus* in H57 on day 13 and *Streptococcus alactolyticus* in control and genus *Lactobacillus* in H57 on day 21. In the caecum

Order RF39 in Control and genus *Ruminococcus* on day 4, *Faecalibacterium prausnitzii* both in Control and H57 on day 13 and family Coriobacteriaceae in control and *Faecalibacterium prausnitzii* on day 21 were the dominant OTUs.

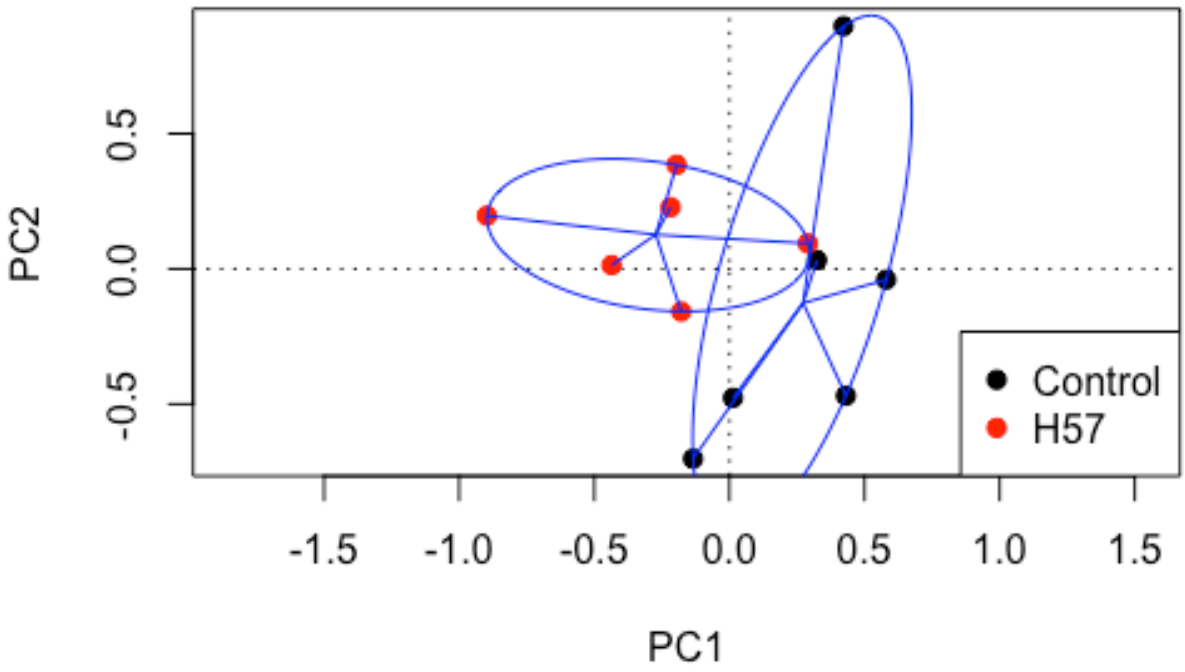


Figure 4-12 Principal Component Analysis (PCA) of normalised relative abundance of OTUs at day 4 from the caecum of chickens fed sorghum based diet.

Relative abundance of several OTUs was significantly different both in the ileum and caecum of Control and H57 treated birds of all three age groups (Table 4-5 and Table 4-6). In the ileum, notable effect was increase of *Lactobacillus* in H57 group on day 21 (5% in Control vs 15.4% in H57). Major OTUs (average relative abundance >1%) altered due to H57 in caecum were genus *Coprococcus* (control = 2.3%, H57 = 7.2%), order Clostridiales (control = 2.8%, H57 = 0.5%), genus Ruminococcus (control = 2.5%, H57 = 0.6%) and genus Oscillospira (control = 1.4%, H57 = 0.5%) on day 4. However, other OTUs with significantly different average relative abundance between Control and H57 were represented in less than 1% relative abundance.

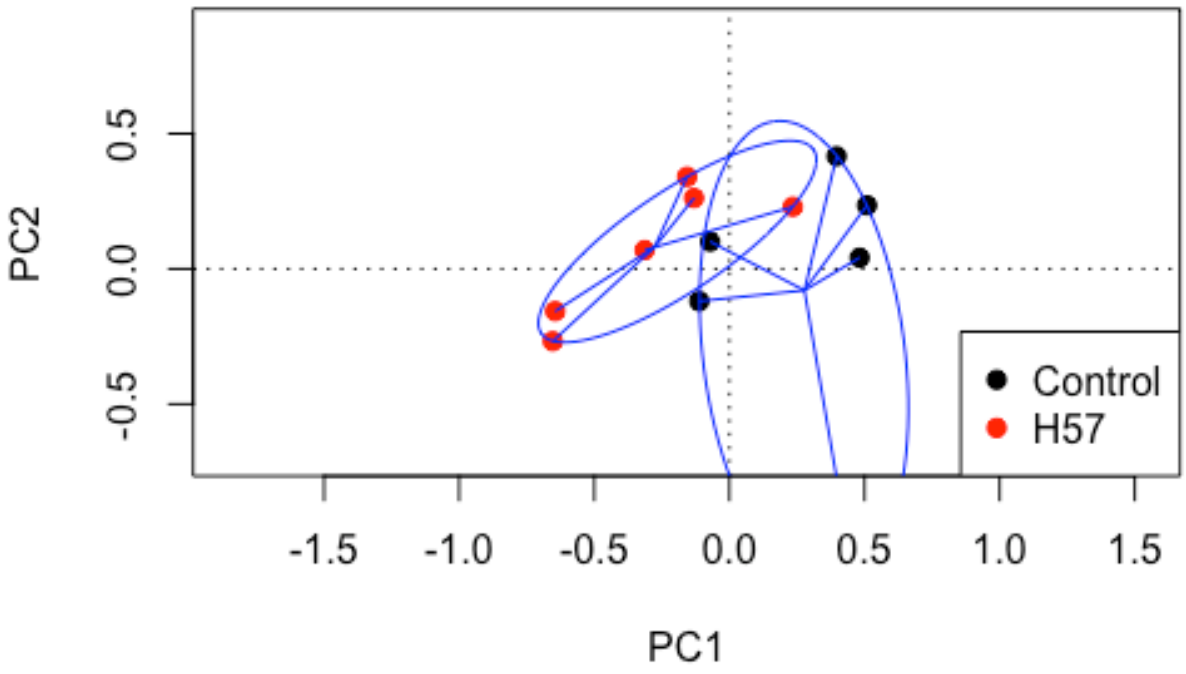


Figure 4-13 Principal Component Analysis (PCA) of normalised relative abundance of OTUs at day 13 from the ileum of chickens fed sorghum based diet.

Table 4-5: Average relative abundances of OTUs significantly altered due to H57 in ileum of birds on sorghum based diets

Age (days)	OTU ID	OTUs	Average relative abundance			P - Value
			Control	H57	Difference (%)	
13	350242	k__Bacteria; p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Lactobacillaceae; g__Lactobacillus; s__	0.88	0.05	-0.83	0.01
	4385535	k__Bacteria; p__Firmicutes; c__Bacilli; o__Bacillales; f__Bacillaceae; g__Bacillus; s__	0.00	1.85	1.84	0.01
21	4414257	k__Bacteria; p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Lactobacillaceae; g__Lactobacillus; s__	4.96	15.36	10.40	0.05

Table 4-6: Average relative normalised abundances of OTUs significantly altered due to H57 in caecum of birds with sorghum based diets

Age (days)	OTU ID	OTUs	Average relative abundance			P-Value
			Control	H57	Difference (%)	
4	132253	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Lachnospiraceae; g__Coprococcus; s__	2.29	7.23	4.94	0.03
	169364	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Lachnospiraceae; g__; s__	0.84	0.22	-0.62	0.05

	2182669	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__; g__; s__	2.78	0.48	-2.30	0.04
	3438642	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Lachnospiraceae; g__Ruminococcus; s__	2.48	0.55	-1.93	0.05
	845900	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Ruminococcaceae; g__Oscillospira; s__	1.38	0.51	-0.87	0.03
13	151870	k__Bacteria; p__Firmicutes; c__Erysipelotrichi; o__Erysipelotrichales; f__Erysipelotrichaceae; g__Coprobacillus; s__	0.28	0.93	0.65	0.01
21	4414257	k__Bacteria; p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Lactobacillaceae; g__Lactobacillus; s__	0.02	0.08	0.06	0.05
	157573	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Rikenellaceae; g__; s__	0.12	0.63	0.51	0.05

4.3.4.2 Wheat based diet

For the chicks raised on the wheat based diet with and without H57, intestinal microbiota were only significantly different between Control and H57 birds in the caecum digesta samples collected on day 21 (P = 0.02, Figure 4-14). There were no significant differences in microbiota structure in either the ileum or caecum between Control and H57 fed birds on day 4 and day 13 and in the ileum on day 21. On day 4, dominant OTUs belonged to the genus *Enterococcus* in the ileum and the family *Lachnospiraceae* in the caecum both in Control and H57 groups. *Streptococcus alactolyticus* and genus *Candidatus Arthromitus* were the dominant OTUs in ileum of control and H57 birds respectively on day 13 while *Faecalibacterium prausnitzii* was the dominant in Control and the genus *Sutterella* in H57 birds in the caecum. Dominant OTUs belonged to the genus *Lactobacillus* in the ileum of both Control and H57 birds on day 21 while *Faecalibacterium prausnitzii* dominated the caecum of the Control and the genus *Megamonas* dominated H57 treated birds.

Addition of H57 to the wheat based diet altered the population of microbes both in the ileum and caecum at day 4, day 13 and day 21 with significant differences in relative abundances of several OTUs *cf*. the Control birds (Table 4-7 and Table 4-8). However, most of these altered OTUs were represented in less than 1% of relative abundance. None of the major OTUs were different in ileum between control and H57 birds. Relative abundances of twenty OTUs were significantly different in caecum between Control and H57 groups on day 13. Out of twenty significantly altered OTUs in caecum, three OTUs were represented in more than 1% of the microbial population. Relative abundance of genus *Lactobacillus* (control = 0.2%, H57 = 1.2%) and genus *Sutterella* (control = 5.5%, H57 = 14.2%) was significantly higher in H57 birds as compared to Control on day 13 while

population of genus *Coprococcus* was increased in H57 gorup as compared to control on day 21 (control = 0.3%, H57 = 1.3%).

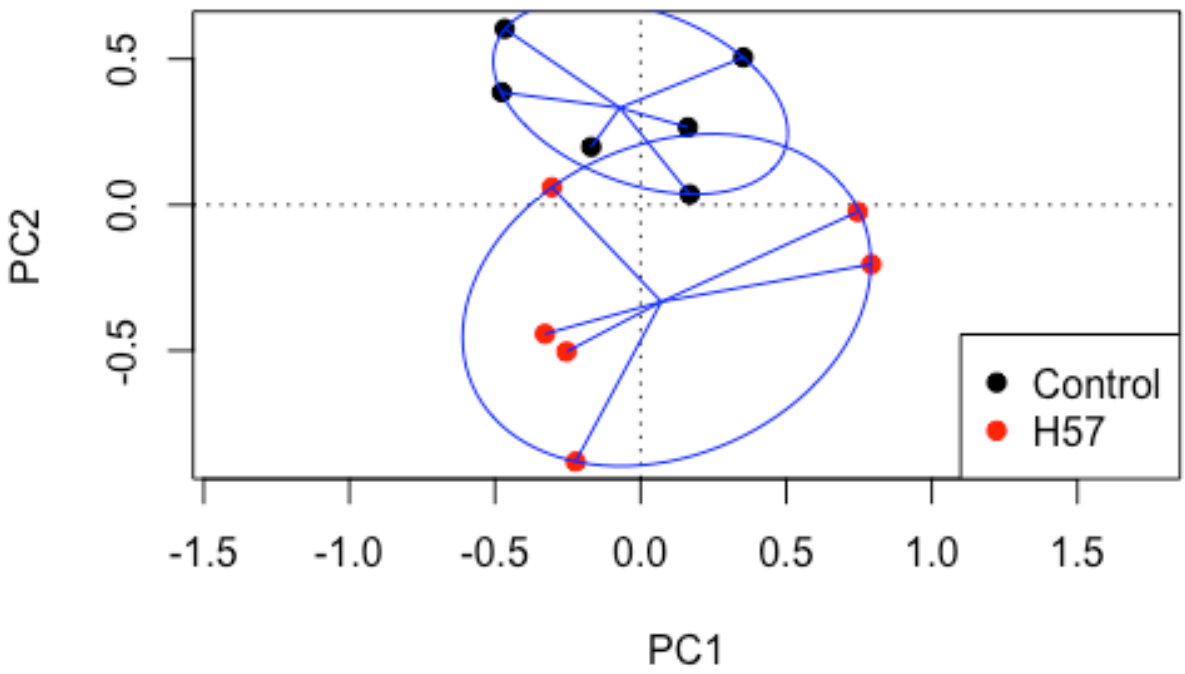


Figure 4-14 Principal Component Analysis (PCA) of normalised relative abundance of OTUs at day 21 from the caecum of chickens fed wheat based diet.

Table 4-7: Average relative abundances of OTUs in the ileum of birds raised on a wheat based diet with H57 supplement which were significantly different to the abundances in Control birds

Age (days)	OTU ID	OTUs	Average relative abundance			P-Value
			Control	H57	Difference (%)	
4	New.ReferenceOTU537	k__Bacteria; p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Lactobacillaceae; g__Lactobacillus; s__	0.09	0.02	-0.07	0.03
21	4385535	k__Bacteria; p__Firmicutes; c__Bacilli; o__Bacillales; f__Bacillaceae; g__Bacillus; s__	0.02	0.19	0.17	0.02

Table 4-8: Average normalised relative abundances of OTUs in the caecum of birds raised on a wheat based diets that were significantly different between birds given H57 and Control birds.

Age (days)	OTU ID	OTUs	Average relative abundance			P-Value
			Control	H57	Difference (%)	
4	539647	k__Bacteria; p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Lactobacillaceae; g__Lactobacillus; s__	0.05	0.21	0.15	0.04
	2182669	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__; g__; s__	0.92	0.08	-0.85	0.03
13	1141398	k__Bacteria; p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Lactobacillaceae; g__Lactobacillus; s__salivarius	0.14	0.87	0.73	0.02
	4362942	k__Bacteria; p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Enterococcaceae; g__Enterococcus; s__	0.01	0.03	0.02	0.03
	350242	k__Bacteria; p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Lactobacillaceae; g__Lactobacillus; s__	0.00	0.02	0.02	0.04
	166911	k__Bacteria; p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Lactobacillaceae; g__Lactobacillus; s__	0.05	0.78	0.74	0.03
	137580	k__Bacteria; p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Lactobacillaceae; g__Lactobacillus; s__	0.00	0.07	0.06	0.04
	176615	k__Bacteria; p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Lactobacillaceae; g__Lactobacillus; s__	0.00	0.03	0.03	0.02
	292057	k__Bacteria; p__Firmicutes; c__Bacilli; o__Lactobacillales; f__; g__; s__	0.00	0.02	0.02	0.02
	4338733	k__Bacteria; p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Lactobacillaceae; g__Lactobacillus; s__reuteri	0.06	0.60	0.54	0.04
	New.Reference OTU742	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Enterobacteriales; f__Enterobacteriaceae; g__; s__	0.00	0.01	0.01	0.01
	1021172	k__Bacteria; p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Lactobacillaceae; g__Lactobacillus; s__salivarius	0.18	1.19	1.00	0.02
	4429986	k__Bacteria; p__Proteobacteria; c__Betaproteobacteria; o__Burkholderiales; f__Alcaligenaceae; g__Sutterella; s__	5.54	14.16	8.62	0.03
	New.Reference OTU670	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Chromatiales; f__; g__; s__	0.00	0.02	0.02	0.03

	211212	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Lachnospiraceae; g__; s__	0.52	0.13	-0.39	0.02
	4433833	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Enterobacteriales; f__Enterobacteriaceae; g__; s__	0.01	0.03	0.03	0.02
	333205	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Ruminococcaceae; g__Ruminococcus; s__	0.22	0.08	-0.14	0.04
21	129401	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Ruminococcaceae; g__Oscillospira; s__	0.19	0.62	0.43	0.03
	2182669	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__; g__; s__	0.03	0.46	0.43	0.02
	3141342	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Lachnospiraceae; g__Coprococcus; s__	0.34	1.31	0.98	0.04
	166637	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Lachnospiraceae; g__Dorea; s__	0.16	0.39	0.23	0.02
	850218	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Veillonellaceae; g__Phascolarctobacterium; s__	0.65	0.06	-0.58	0.02

4.3.4.3 Sorghum and wheat blend diet

Supplementing a diet based on a blend of sorghum and wheat with H57 resulted in significant differences in the microbial populations in the caecum of Control *vs* H57 treated birds at day 13 (P = 0.006, Figure 4-15). Microbiota structure of all other age group and sites were similar between Control and H57. Genus *Enterococcus* in ileum on day 4, genus *Lactobacillus* in caecum on day 4, family *Clostridiaceae* in ileum on day 13 and *Faecalibacterium prausnitzii* in caecum on day 13 dominated both in control and H57 birds. At day 21 *Lactobacillus* were the dominant OTUs in the ileum of both Control and H57 birds while *Faecalibacterium prausnitzii* in Control and the genus *Bacteroides* in H57 groups were the dominant OTUs respectively in caecum.

Although there were some OTUs with significantly different relative abundance between Control and H57 none of the OTUs with more than 1% of relative abundance were significantly different between two groups (Table 4-9). In caecum, reduction of genus *Ruminococcus* on day 4 and reduction of *Faecalibacterium prausnitzii* on day 13 in H57 group as compared to Control were the major changes (Table 4-10).

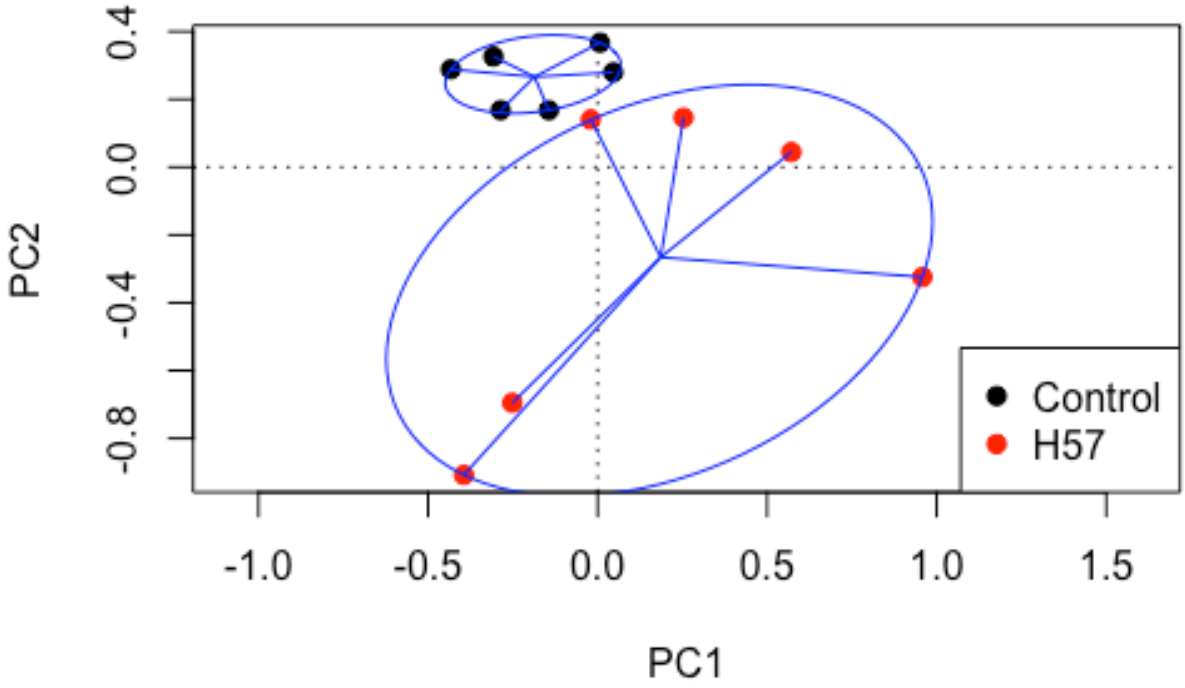


Figure 4-15 Principal Component Analysis (PCA) of normalised relative abundance of OTUs at day 13 from the caecum of chickens fed the diet based on sorghum and wheat blend.

Table 4-9: Average normalised relative abundances of OTUs significantly altered in the ileum of birds raised on a sorghum and wheat blend diet when supplemented with H57.

Age (days)	OTU ID	OTUs	Average relative abundance			P-Value
			Control	H57	Difference (%)	
4	4385535	k__Bacteria; p__Firmicutes; c__Bacilli; o__Bacillales; f__Bacillaceae; g__Bacillus; s__	0.00	0.30	0.29	0.03
13	New.ReferenceOTU410	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__; g__; s__	0.00	0.01	0.01	0.02
	4385535	k__Bacteria; p__Firmicutes; c__Bacilli; o__Bacillales; f__Bacillaceae; g__Bacillus; s__	0.00	0.77	0.77	0.02
21	4385535	k__Bacteria; p__Firmicutes; c__Bacilli; o__Bacillales; f__Bacillaceae; g__Bacillus; s__	0.00	0.19	0.19	0.01
	4334055	k__Bacteria; p__Firmicutes; c__Bacilli; o__Lactobacillales; f__; g__; s__	0.18	0.02	-0.16	0.02

Table 4-10: Average normalised relative abundances of OTUs significantly altered in the caecum of birds raised on a sorghum and wheat blend diet when supplemented with H57.

Age (days)	OTU ID	OTUs	Average relative abundance			P-Value
			Control	H57	Difference (%)	
4	157516	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Lachnospiraceae; g__Ruminococcus; s__	2.98	0.88	-2.10	0.03
	3082155	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Lachnospiraceae; g__Ruminococcus; s__	3.53	0.53	-3.00	0.03
13	574528	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Clostridiaceae; g__; s__	1.44	0.18	-1.26	0.02
	New.ReferenceOTU903	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Clostridiaceae; g__; s__	0.12	0.00	-0.12	0.04
	137580	k__Bacteria; p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Lactobacillaceae; g__Lactobacillus; s__	0.03	0.01	-0.02	0.04
	157297	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Ruminococcaceae; g__Faecalibacterium; s__prausnitzii	20.49	7.96	-12.54	0.01
	157224	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Ruminococcaceae; g__Faecalibacterium; s__prausnitzii	29.02	15.67	-13.35	0.04
21	4447432	k__Bacteria; p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Lactobacillaceae; g__Lactobacillus; s__	0.21	0.98	0.77	0.04

4.3.5 Experiment 3; effect of H57 on faecal microbiota

In Experiment 3, the microbial profile of faeces was determined in the sample collected at age day 13. Overall there was a significant difference (P = 0.019) in the microbial profile in faeces between Control and H57 treated birds in the group fed a wheat based diet but there was no difference in the group fed a sorghum based diet or a mix of wheat and sorghum (P>0.05).

The dominant OTU in the wheat diet was *Streptococcus alactolyticus* in the Control and *Lactobacillus* in the H57 treatment while in the sorghum diet *Streptococcus alactolyticus* was dominant in both the Control and H57. For the mixed diet *Lactobacillus* was the dominant OTU in the Control group and *Streptococcus alactolyticus* in the H57 group. Relative abundance of six OTUs was significantly different between Control and H57 in the wheat based diet (Table 4-11) while there were three OTUs with significantly different relative abundance in sorghum diet (Table

4-12) There was no difference in the relative abundance of OTUs between Control and H57 for the mixed diet except for a very minor OTU representing *Bacteroides plebeius* (0.0004% in control and nil in H57).

Table 4-11 Experiment 3. OTUs with significantly different relative abundance between Control and H57 in the faeces of chicks at day 13 fed a wheat based diet

OTU_ID	OTUs	Average relative		P-value
		Control	H57	
New.ReferenceOTU742	f__Enterobacteriaceae; g__; s__	0.17	0.65	0.04
4473883	g__Streptococcus; s__alactolyticus	30.79	2.72	0.01
4447567	o__Lactobacillales; f__; g__; s__	1.46	0.24	0.04
New.ReferenceOTU286	o__Chromatiales; f__; g__; s__	0.52	2.41	0.02
4337090	g__Streptococcus; s__	0.89	0.03	0.02
New.ReferenceOTU156	g__Streptococcus; s__alactolyticus	0.57	0.04	0.02

Table 4-12 Experiment 3. OTUs with significantly different relative abundance between Control and H57 in the faeces of chicks at day 13 fed a sorghum based diet.

OTU_ID	OTUs	Average relative abundance		P-value
		Control	H57	
16195	g__Candidatus Arthromitus; s__	0.14	1.06	0.04
4447567	o__Lactobacillales; f__; g__; s__	1.34	0.34	0.04
New.ReferenceOTU156	g__Streptococcus; s__alactolyticus	0.42	0.05	0.03

4.3.6 Correlation between body weight of the chickens and microbial profile in experiment 3

There was significant correlation (positive or negative) between body weight and relative abundance of OTUs for some OTUs but not for all OTUs that are significantly different between Control and H57. For sorghum based diet, there were 11 OTUs (day 4 caecum = 5, day 13 ileum = 2, day 13 caecum = 1, day 21 ileum = 1, day 21 caecum = 2) significantly different between Control

and H57. Out of these 11 OTUs, 4 OTUs (day 13 ileum = 2, day 13 caecum = 1, day 21 ileum = 1) were significantly correlated (positive or negative; Table 4-13, Figure 4-16, Figure 4-17, Figure 4-18, Figure 4-19, and Figure 4-20) with body weight. For the wheat based diet, there were 24 OTUs (day 4 ileum = 1, day 4 caecum = 2, day 13 caecum = 15, day 21 ileum = 1, day 21 caecum = 5) significantly different between Control and H57. Out of these 24 OTUs, 16 OTUs (day 4 ileum = 1, day 4 caecum = 1, day 13 caecum = 14) were significantly correlated (positive or negative; Table 4-13, Figure 4-21, Figure 4-22, Figure 4-23) with body weight. Notably, there was no correlation between body weight and relative abundance of OTUs when there was no significance difference of body weight between Control and H57 i.e. there was no correlation between any of OTUs and body weight in wheat and sorghum mix diet even if there were 13 OTUs (day 4 ileum = 1, day 4 caecum = 2, day 13 ileum = 2, day 13 caecum = 5, day 21 ileum = 2, day 21 caecum = 1) significantly different between Control and H57. Monotonic relationship between relative abundance of OTUs and body weight of chickens (showing correlation coefficient and P value) for OTUs which have relative abundance of >1% at least in a sample and significantly correlated with body weight are shown in Figure 4-16 to 4-23 and Table 4-13.

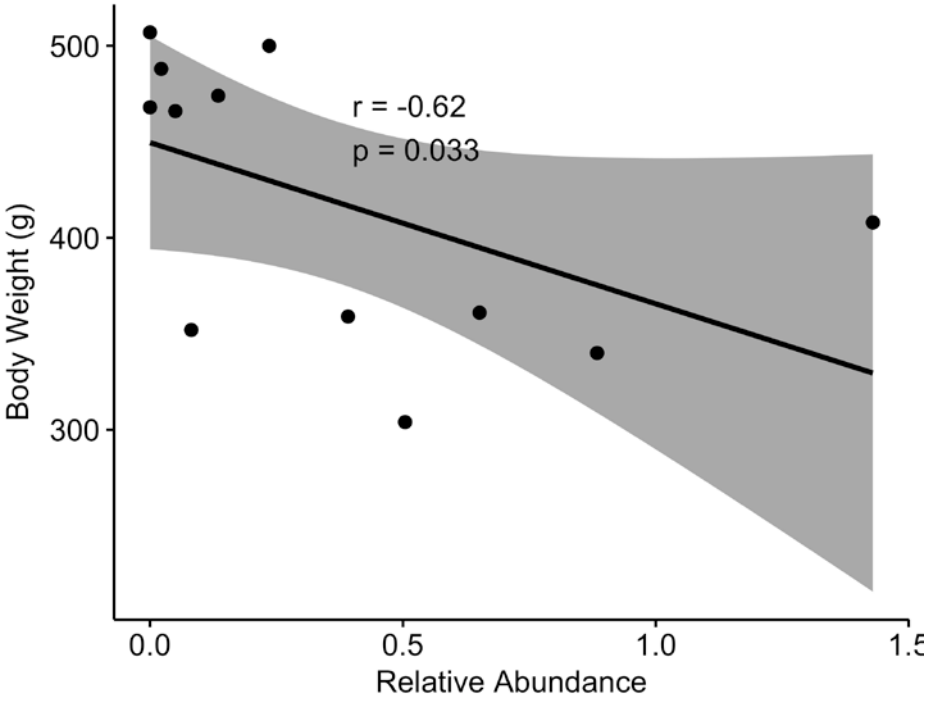


Figure 4-16: Correlation between body weight and relative abundance of g_Lactobacillus (OTU ID 539647) in ileum of birds with sorghum based diet at age day 13.

Table 4-13 Correlation between body weight and relative abundance of OTUs, which were significantly different between control and H57 treated chickens. Positive corelation coefficients indicate positive correlation while negative correlation coefficients indicate negative correlation.

Diet	Age (day)	Site	OTU	Correlation coefficient	P-Value
Sorghum	13	Ileum	*Lactobacillus (*OTU ID 539647)	-0.62	0.033
Sorghum	13	Ileum	*Lactobacillus (*OTU ID 350242)	-0.71	0.01
Sorghum	13	Ileum	*Bacillus (*OTU ID 4385535)	0.77	0.0036
Sorghum	13	Caecum	Copro*bacillus (*OTU ID 151870)	0.78	0.0047
Sorghum	21	Ileum	*Lactobacillus (*OTU ID 4414257)	0.61	0.034
Wheat	13	Caecum	*Lactobacillus (*OTU ID 166911)	0.76	0.0059
Wheat	13	Caecum	*Lactobacillus (*OTU ID 4447432)	0.67	0.02
Wheat	13	Caecum	*Ruminococcus gnavus (*OTU ID 68842)	0.70	0.011

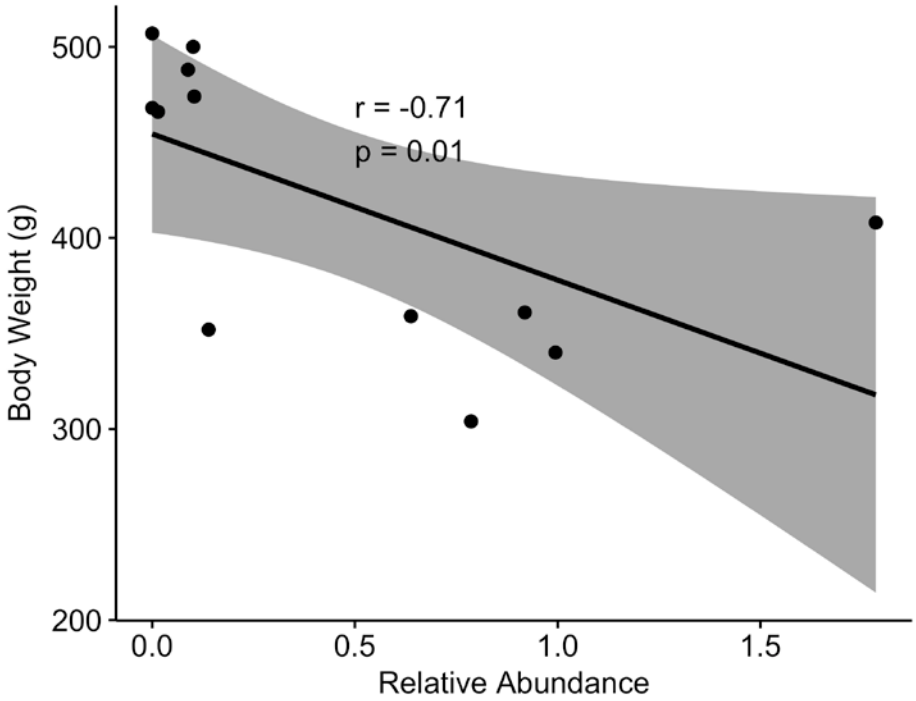


Figure 4-17: Correlation between body weight and relative abundance of g_*Lactobacillus* (OTU ID 350242) in ileum of birds with sorghum based diet at age day 13.

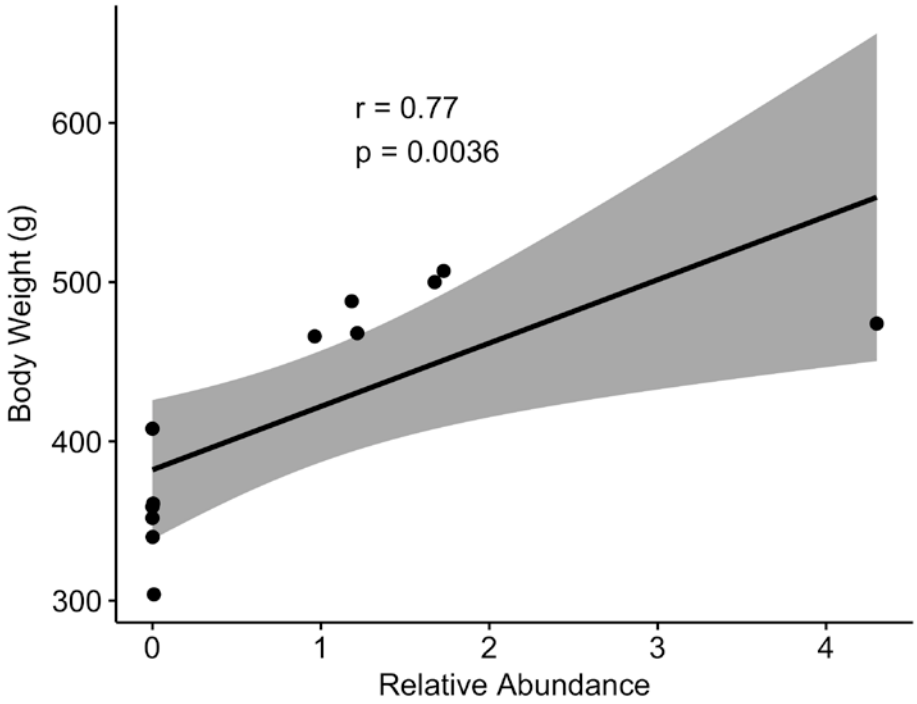


Figure 4-18: Correlation between body weight and relative abundance of g_*Bacillus* (OTU ID 4385535) in ileum of birds with sorghum based diet at age day 13.

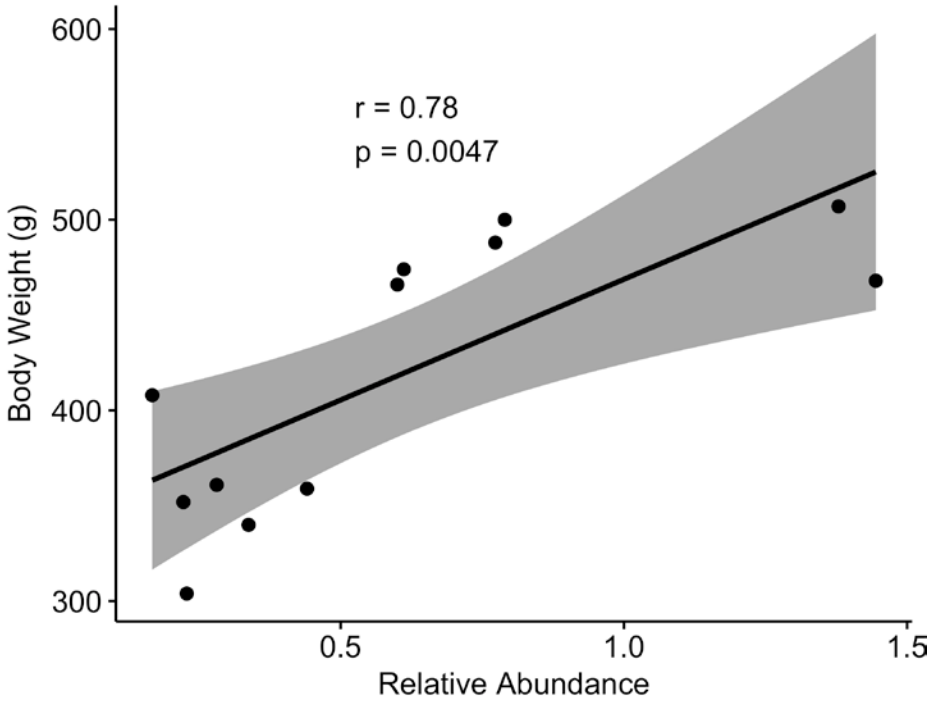


Figure 4-19: Correlation between body weight and relative abundance of g_Copro*bacillus (*OTU ID 151870) in caecum of birds with sorghum based diet at age day 13.

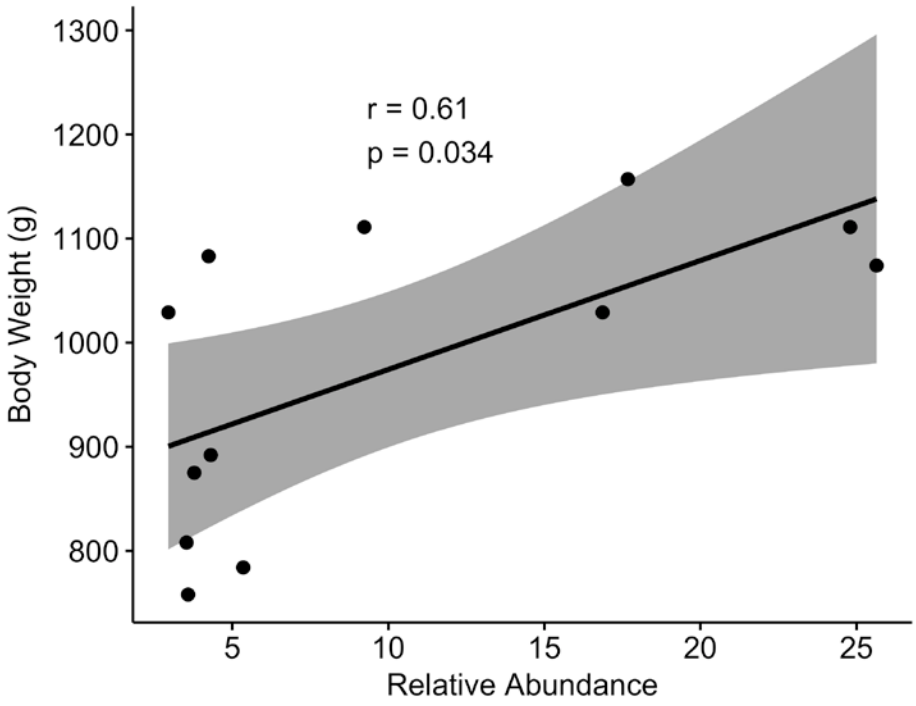


Figure 4-20: Correlation between body weight and relative abundance of g_*Lactobacillus (*OTU ID 4414257) in ileum of birds with sorghum based diet at age day 21.

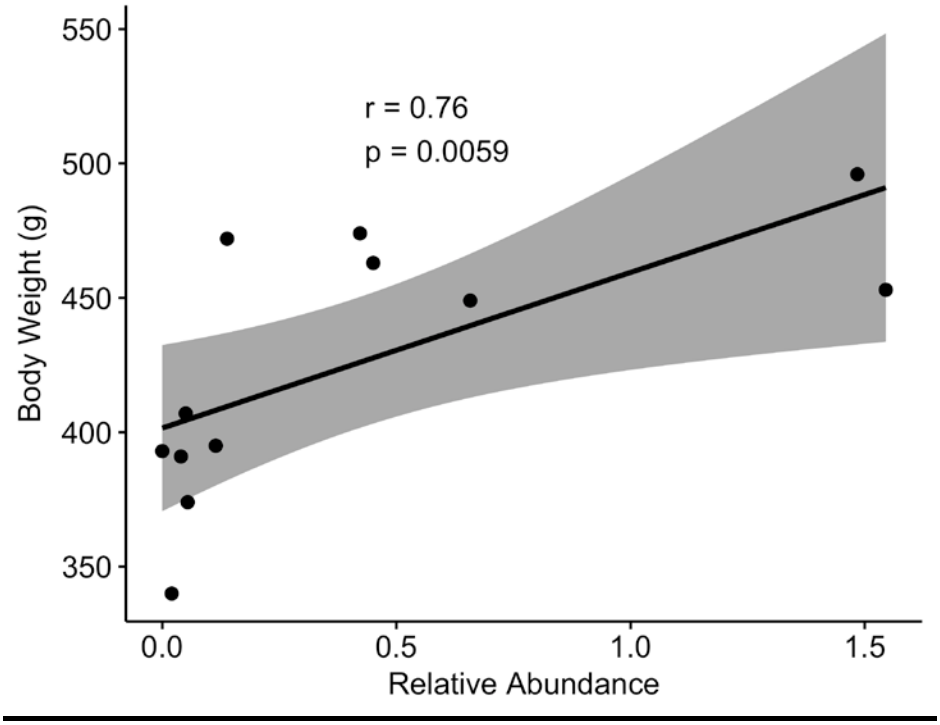


Figure 4-21: Correlation between body weight and relative abundance of g_*Lactobacillus* (OTU ID 166911) in caecum of birds with wheat based diet at age day 13.

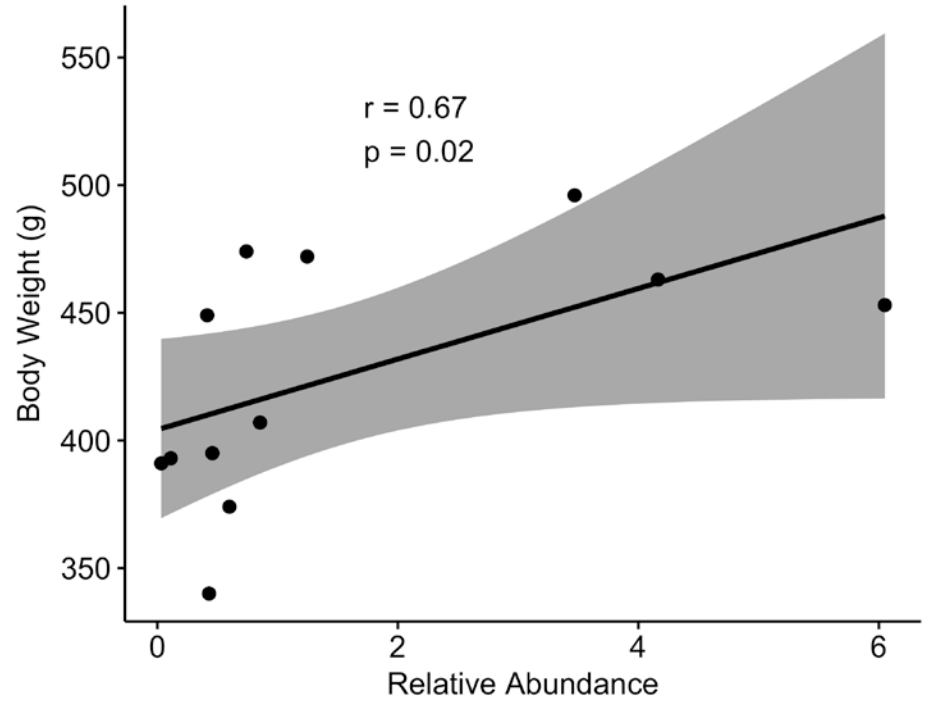


Figure 4-22: Correlation between body weight and relative abundance of g_*Lactobacillus* (OTU ID 4447432) in caecum of birds with wheat based diet at age day 13.

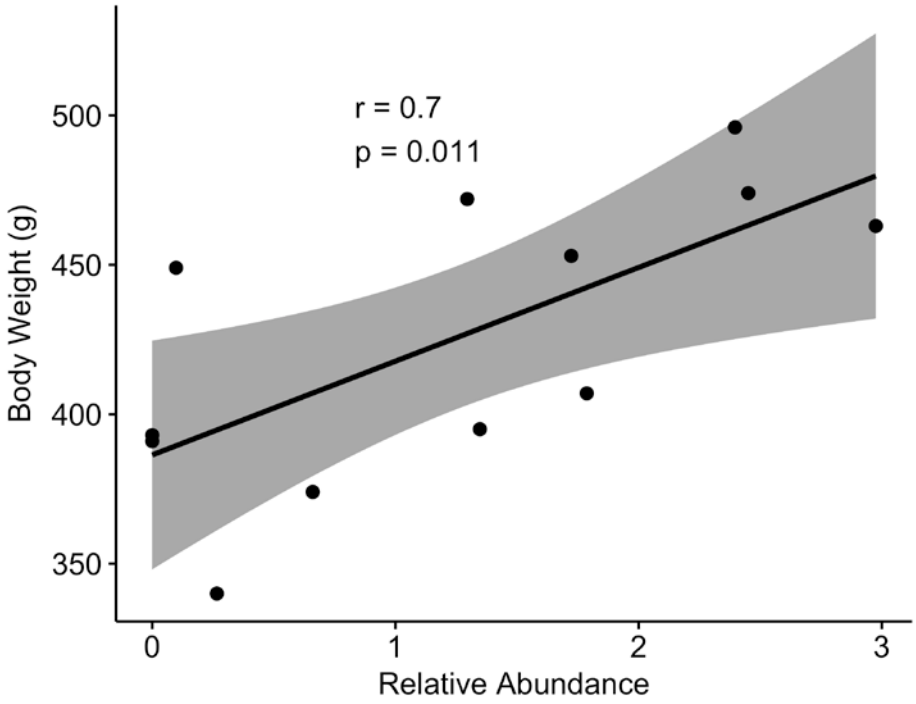


Figure 4-23: Correlation between body weight and relative abundance of g_*Ruminococcus* s_*gnavus* (OTU ID 68842) in caecum of birds with wheat based diet at age day 13.

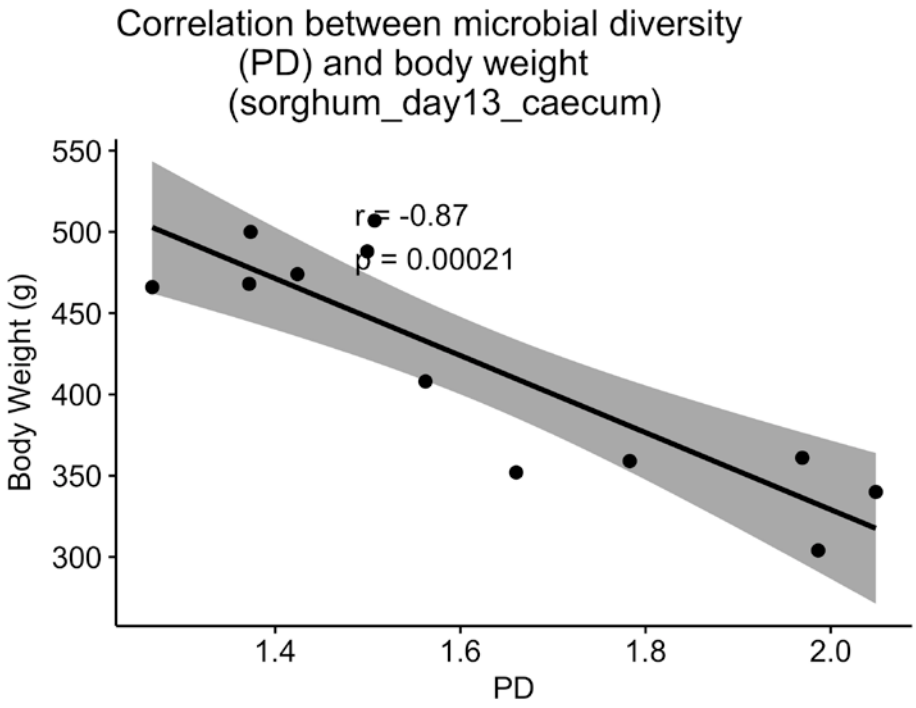
4.3.7 Correlation between body weight of the chickens and microbial diversity in experiment 3

Although correlation between body weight of the individual birds and the phylogenetic diversity (PD) was not consistent with feed (sorghum, wheat and mix), site (ileum and caecum) and age of the birds (day 4, 13 and 21), however, in general it appears that diversity is negatively correlated with body weight (growth rate). The body weight was negatively correlated with microbial diversity at day 13 and 21 for sorghum based diet, at day 4 for wheat based diet and at day 13 for mixed diet (Table 4-14 and Figure 4-24).

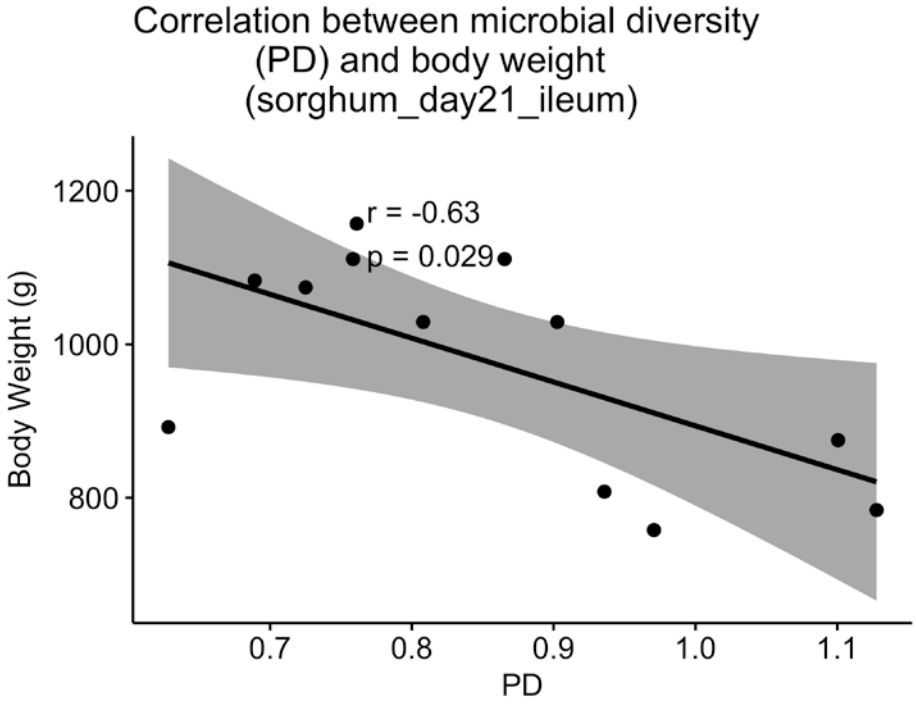
Table 4-14 Correlation between body weight and microbial diversity (phylogenetic diversity) between control and H57 treated chickens. Positive correlation coefficients indicate positive correlation while negative correlation coefficients indicate negative correlation.

Diet	Age (day)	Site	Correlation coefficient	P-Value
Sorghum	13	Caecum	-0.87	0.0002
Sorghum	21	Ileum	-0.63	0.029
Wheat	4	Caecum	-0.71	0.01
Mix	13	Caecum	-0.69	0.013

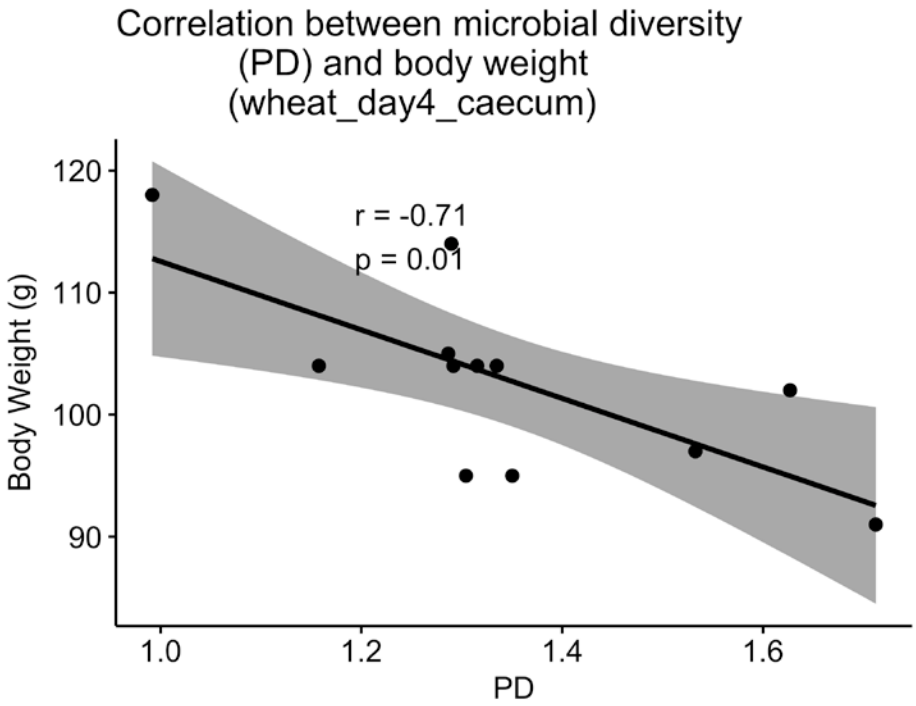
a)


Correlation between microbial diversity
(PD) and body weight
(sorghum_day13_caecum)
r = -0.87
p = 0.00021
Body Weight (g)
550
500
450
400
350
300
1.4
1.6
1.8
2.0
PD

b)


Correlation between microbial diversity
(PD) and body weight
(sorghum_day21_ileum)
r = -0.63
p = 0.029
Body Weight (g)
1200
1000
800
0.7
0.8
0.9
1.0
1.1
PD

c)



d)

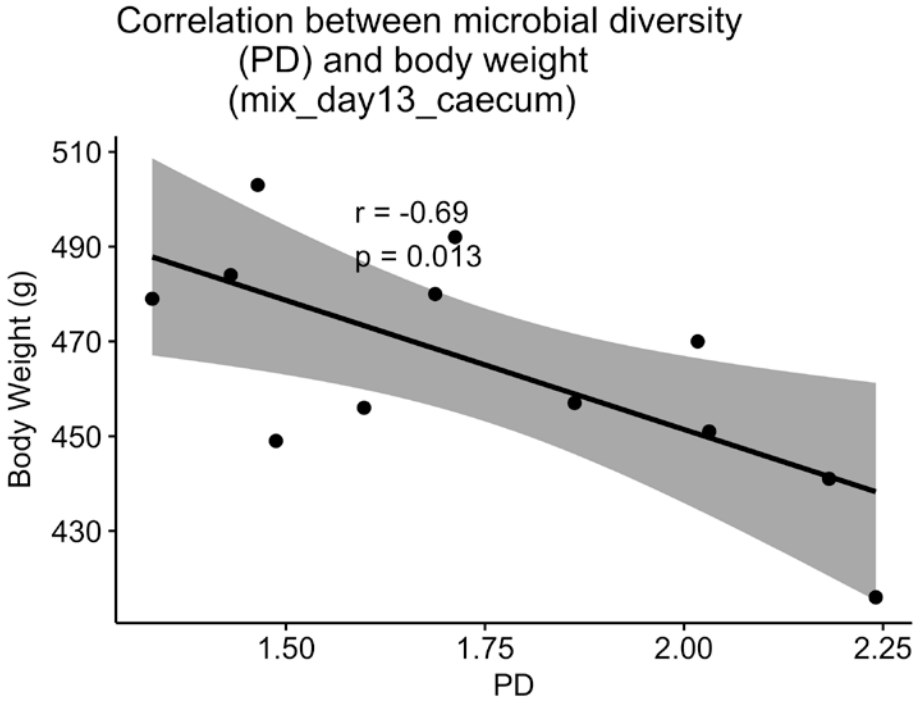


Figure 4-24 Correlation between body weight and microbial diversity. a) sorghum, day 13, caecum b) soghum, day 21, ileum c) wheat, day 4, caecum and d) mix, day 13, caecum

4.4 Discussion

4.4.1 Effects of H57 on the intestinal microbiota and correlation with body weight

Bacillus amyloliquefaciens H57 modified both the ileal and caecal microbial community structure of the broiler chicken. However, the effect of H57 on intestinal microbiota was not uniform across all the experiments, diet (sorghum, wheat or mixed) and age of the birds. We have found significant batch-to-batch difference in the intestinal microbial community but it is difficult to make the inference about how this difference relates to overall microbial function in the gut.

Although there were several microbes significantly different between Control and H57 groups, there was significant correlation (positive or negative) between body weight and relative abundance of OTUs for some OTUs but not for all OTUs which are significantly different between Control and H57. Therefore, some of the OTUs might have directly contributed to the differences in the body weight while other may not have directly influence on the body weight even if there were significantly different abundance between Control and H57.

4.4.1.1 Lactobacillus could be an important member of the intestinal microbiota determining birds performance

Lactobacillus spp. are the most consistently altered bacterial species in our study. By doing a comprehensive meta analysis of 92 different studies in humans, domestic food animals and experimental models; Million *et al.* (2012a) concluded that *Lactobacillus acidophilus, Lactobacillus fermentum* and *Lactobacillus ingluviei* were associated with weight gain in poultry while *Lactobacillus plantarum* and *Lactobacillus gasseri* were associated with weight loss in human and animals. In our study, relative abundance of OTUs representing *Lactobacillus salivarius* and *Lactobacillus spp.* were significantly higher in the ileum of H57 treated birds in experiment 1. By contrast, *L. salivarius* has been suggested to be associated with growth depression in broilers through their effect on bile salts in a previous study (Ranjitkar et al., 2015). However, effect of H57 on *Lactobacillus* is variable in experiment 3. Relative abundance of *Lactobacillus spp*. is reduced on day 4 – ileum (both in sorghum and wheat based diet) and increased on day 13 – caecum (wheat) and day 21 – caecum (both in sorghum and wheat based diet). Similarly, relative abundance of *L. salivarius* and *Lactobacillus reuteri* was increased on day 13 caecum on wheat based diet. Million et al. (2012b) have also reported that increased concentration of *Lactobacillus reuteri* in the gastrointestinal microbiota is associated with weight gain. *Lactobacillus* spp. have been shown to have diverse genetic and functional characteristics with species specific metabolic functions

(Million et al., 2012a). This could be related to the variable results in our experiments. We have found both positive and negative correlation of OTUs representing *Lactobacillus* with body weight (Figure 4-17, Figure 4-20, Figure 4-21, Figure 4-22).

4.4.1.2 Ratio of bacteroidetes and firmicutes

Previous studies have revealed that population concentration of the bacterial phylum bacteroidetes and firmicutes are associated with body weight in human and mice. Several studies have found reduced concentration of bacteroidetes is associated with weight gain in human and mice (Ley et al., 2006, Turnbaugh et al., 2009, Armougom et al., 2009, Santacruz et al., 2009, Zuo et al., 2011, Ley et al., 2005). In contrast, some other studies have reported the opposite results as higher concentration of bacteroidetes (Collado et al., 2008, Schwiertz et al., 2010) and lower concentration of firmicutes (Schwiertz et al., 2010) associated with weight gain. Mai *et al*. (2009) found no difference in bacteroidetes population between obese and lean individuals. In experiment 1, population of bacteroidetes were significantly increased while firmicutes and actinobacteria were reduced in caecum. Colonization of the intestine with *Bacteroides thetaiotaomicron* and *Methanobrevibacter smithii* has shown to increase energy extraction from the diet in mice (Samuel and Gordon, 2006). Similarly, Intestinal microbiota consortium of obese mice has been shown to be more efficient to harvest energy from the diet as compared to microbiota of lean mice (Turnbaugh et al., 2006). In contrast, firmicutes were significantly higher in day 4 (both in ileum and caecum) in experiment 3 with the sorghum based diet. In experiment 3 with the sorghum based diet at day 13, relative abundance of bacteroidetes were significantly higher and that of firmicutes were significantly lower in H57 treated group. For wheat based diet (experiment 3), there was no difference in bacteroidetes or firmicutes instead proteobacteria was significantly lower on day 13 but reversed (significantly higher) on day 21 in caecum of H57 treated birds.

4.4.1.3 Structure of inherent resident microbes may determine the effects of H57

The variability in results poses difficulty in elucidating the possible mode of action of H57 as a probiotic. The effects of H57 on growth, feed intake and feed conversion varied with the experiment (chapter 3) as with the effect on the intestinal microbiota. It could therefore be reasonable to assume that there may be correlation between inherent resident microbiota in the intestine and how H57 affects this microbiome and ultimately the performance of the chickens. If the inherent resident microbiome in the chickens was different in different batches of chickens then this may influence the impact that H57 is able to exert on that microbiome.

Due to intensification of the poultry industry, chicks are hatched in a relatively sterile environment. Therefore, microbes colonizing the GI tract depend mainly on the initial external environment (both in hatchery and the rearing farm) that they are exposed to and the feed and water. To investigate whether this hypothesis is feasible, the microbial profile of the ileum and caecum of Control group birds of the same age (21 days) from experiment 1 and experiment 3, fed a similar diet (sorghum based diet) was ordinated by Detrended Correspondence Analysis (Figure 4-25 and Figure 4-26). The inherent resident microbial profiles between these two experiments, both in ileum and caecum, were significantly different (PERMANOVA, P = 0.001) and clustered separately as shown in Figure 4-25 and Figure 4-26.

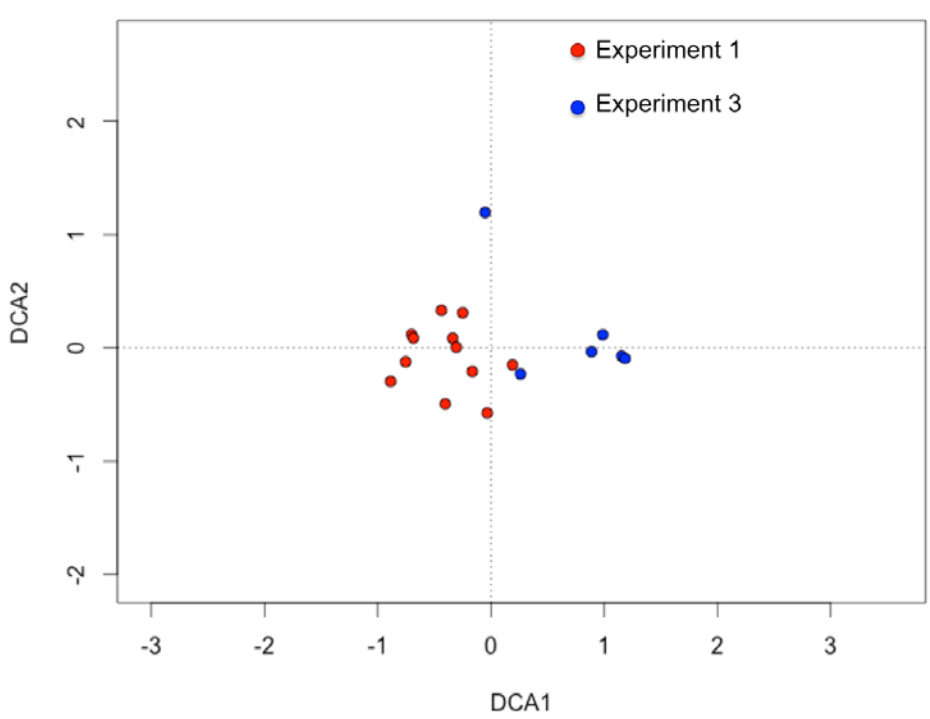


Figure 4-25: Detrended Correspondence Analysis (DCA): Comparison of microbial profile in the ileum of birds from experiment 1 and experiment 3 both fed sorghum based Control diets. Between the two experiments the microbial profiles at genus level are significantly different (p=0.001).

The significantly different microbial profiles at day 21 between the birds of two experiments both fed similar diets suggests that the colonization of the intestine in these two groups of birds started with two different sets of “seed microbes” at and shortly after hatching. Therefore, we presume that the batch of the chicks could be a determining factor on the initial structure of the gastrointestinal microbiota colonising the gastrointestinal tract.

Most of the studies about the effects of probiotics on the productivity and health of domestic animals and poultry have drawn their conclusions based on single experiments and the effect of probiotics have been reported quite variable and inconsistent (FAO, 2016). This variability of the

effects of probiotics could be the reflection of underlying variability in the gastrointestinal microbial structure, as we've seen in our study. This apparent lack of consistency of the microbiome composition over time and changes with treatment has been found in other studies based on DNA sequencing (Stanley et al., 2016, Stanley et al., 2013).

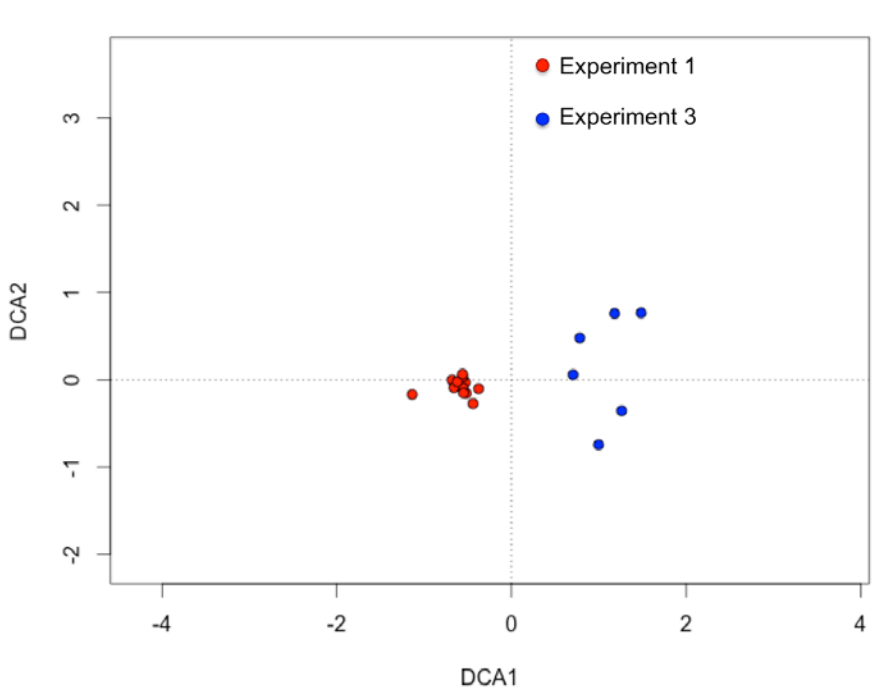


Figure 4-26: Detrended Correspondence Analysis (DCA): Comparison of microbial profile in the caecum of birds from experiment 1 and experiment 3 both fed sorghum based Control diets. Between the two experiments the microbial profiles at genus level are significantly different (p=0.001)

As discussed previously (chapter 2), H57 had been effective in improving the growth rate when the chickens were underperforming but not in the chickens growing optimally. In this context, we presume that some species within the intestinal microbiota could be responsible for the depression of growth in chickens and H57 may modify the intestinal microbial profile in a way that limits these species and overcomes growth depression.

As there was no consistent effect on H57 on the GIT microbes, it appeared here that H57 either assists one of several beneficial microbial strains to establish (flourish) in the gut or reduces the populations of one or several growth depressing strains. Alternatively, H57 could be altering the GIT microbiota at a functional level rather than at the taxonomic level. To support this assumption, the effect of H57 on the overall microbial profile was significant in experiment 1 but the overall effect was less prominent in experiment 3. In contrast, the effect of H57 on growth rate was more prominent in experiment 3 than in experiment 1. To investigate possible functional variability between microbiomes, a metagenomic analysis of samples from experiment 3 was undertaken (**chapter 5**).

Chapter 5 Metagenomic analysis of the caecal microbiome in response to Bacillus amyloliquefaciens H57

5.1 Introduction

As described in chapters 3 and 4, H57 improved the growth performance of broiler chickens and modified the gastrointestinal microbial populations. However, improvement in growth rate occurred both with and without significant effects on caecal microbial populations. Therefore, it appears that the phenotypic response to H57 is due to its effect on the underlying microbial functional activity rather than the microbial diversity (OTU) profile *per se*. This chapter analyses the effect of H57 on potential microbial functions in the caeca.

While phylogenetic marker gene sequencing (chapter 4) indicates 'which microbes are where and at what abundance' (Muyzer et al., 1993, Lane et al., 1985), it does not provide detailed insight into a microbiome's functional capabilities. To address this a metagenomic analysis of the GIT was undertaken, *i.e.* direct shotgun sequencing of fragmented genomic DNA extracted from environmental samples (Venter et al., 2004). This approach facilitates identification and enumeration of the protein coding genes encoded within the genomes of the populations present in each sample, allowing inferences to be made about the community's metabolic capabilities (Riesenfeld et al., 2004).

The biggest difference in body weight between Control and H57 treated birds was on day 14 in the experiment 3 (chapter 3). Therefore samples collected from the caeca of birds at day 13 were chosen for metagenomic analysis. In addition, the microbial populations were significantly different between sorghum and wheat based diets. Therefore, samples from both sorghum fed and wheat fed chickens were selected for further study. As there was no effect of H57 on the sorghum plus wheat mixed diet, no samples were chosen for shotgun sequencing from these birds. The microbial population per unit digesta weight and the diversity are the largest in the caeca when compared with other parts of the gastrointestinal tract in chickens (Bjerrum et al., 2006, Sergeant et al., 2014, Apajalahti et al., 2004). The caecum is the most important and primary site for microbial fermentation of undigested carbohydrate into short chain fatty acids (Józefiak et al., 2004).

5.2 Materials and methods

5.2.1 Experimental design and samples

Metagenomic shotgun sequencing was undertaken with a subset of samples from experiment 3 (chapter 3). A total of 20 caecal digesta samples from day 13 were randomly selected for metagenomic shotgun sequencing; five samples each for Sorghum Control, Sorghum H57, Wheat Control and Wheat H57.

Samples were collected and DNA was extracted as described in chapter 4.

5.2.2 Preparation of metagenomic library and sequencing

The concentration of genomic DNA was measured using a Qubit 2.0 fluorometer (Thermo Fisher Scientific Inc, Victoria, Australia) with a dsDNA Broad Range Assay kit (Thermo Fisher Scientific Inc, Victoria, Australia) and diluted with ultrapure distilled water to 5 ng μl^{-1}. A paired-end indexed library was prepared by using the Illumina Nextera DNA Library Preparation Kit (Illumina, San Diego, CA, USA) as per the manufacturer's instructions. Genomic DNA (10 µl) was fragmented and tagged with adaptor sequences using the transposome enzyme (Tagment DNA Enzyme). Tagmentation (fragmentation and tagging) of genomic DNA was achieved by mixing 25 µl of Tagment DNA Buffer and 5 µl of Tagment DNA Enzyme with genomic DNA (50 ng in total) and running in a thermal cycler at 55°C for 5 minutes.

Tagmented DNA was purified by spinning down the tagmented DNA solution mixed with 180 µl Zymo DNA binding buffer (Zymo Research, Irvine, CA, USA) through a Zymo-Spin I-96 Plate (Zymo Research, Irvine, CA, USA). DNA in the Zymo-Spin Plate was washed with 300 µl of Zymo Wash) and eluted with 25 µl of Resuspension Buffer (Zymo Research, Irvine, CA, USA).

Limited cycle PCR was then used to amplify the purified tagmented DNA and add index adaptors Index 1 (i7), Index 2 (i5), sequencing adaptor and common adaptors (P5 and P7) (Nextera XT Index Kit, Illumina, San Diego, CA, USA). After arranging Index 1 and Index 2 adaptors in TruSeq Index Plate Fixture (Illumina, San Diego, CA, USA), PCR reaction plate was prepared by mixing 5 µl each of i7 adaptor, i5 adaptor and PCR Primer Cocktail, 15 µl of Nextera PCR Master Mix (Illumina, San Diego, CA, USA) and 20 µl of purified tagmented DNA. DNA was amplified by PCR with reaction parameters of 72°C for 3 minutes, 98°C for 30 seconds, 5 cycles of 98°C for 10 seconds, 63°C for 30 seconds and 72°C for 3 minutes and finally holding at 10°C.

Agencourt AMPure XP beads (Beckman Coulter Australia Pty Ltd, Lane Cove, NSW, Australia) were used to clean DNA amplified as above. AMPure XP beads (30 µl) were added to 50 µl of PCR products and incubated at room temperature for 5 minutes. The samples were kept in magnetic stand and the supernatant was discarded after it was clearly separated from the beads (~2 minutes). The beads with DNA were washed twice with 200 µl freshly prepared 80% ethanol without disturbing the beads. The samples were then removed from the magnetic stand after air-drying for 15 minutes and the DNA was resuspended in 32.5 µl of Resuspension Buffer and placed again in magnetic stand for 2 minutes to clear the liquid. The clean supernatant (amplified and cleaned DNA) then underwent quality control analysis to check the size distribution of the DNA library by running 1 µl of 1:3 diluted (with ultrapure water) library on an Agilent Technology 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

Concentrations of DNA in DNA library templates were measured using a Qubit fluorometer (Thermo Fisher Scientific Inc, Victoria, Australia) and normalized to 2 nM using Tris-Cl 10 mM, pH 8.5 with 0.1% Tween 20. Normalized DNA samples were pooled, denatured and diluted to 1.8 pM and sequenced using the Illumina NextSeq 500 sequencing platform (Illumina, San Diego, CA, USA).

5.2.3 Sequence processing and analysis

5.2.3.1 Gene centric analysis

Shotgun sequence reads were aligned with protein reference database Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa and Goto, 2000) using double index alignment of next-generation sequencing data (DIAMOND) program (Buchfink et al., 2015) to assign shotgun reads to a functional gene. The gene abundance table obtained from DIAMOND was annotated with KEGG Orthology (KO) annotations using Client-side REST access to KEGG (KEGGREST) (Tenenbaum, 2013) package in R (R Core Team, 2016).

The resulting KO gene count matrix with each row representing KO identifier and each column representing samples, was then analysed with the DESeq2 package (Love et al., 2014) in R to find differentially abundant genes between treatments (Control and H57). The DESeq2 package is generally used to analyse differential expression of genes from RNA-seq data. We used the package to analyse differential abundances of genes between treatments. DESeqDataSet, the data object class for DESeq2 package, was prepared separately for sorghum and wheat based diets by using the function DESeqDataSetFromMatrix() with associated “design formula” testing the effect of

treatment (Control and H57). Control was made the first level in the treatment factor with function relevel() to ensure that the default log2 fold change would be calculated as H57 over the Control.

Variance-stabilizing transformation (Lin et al., 2008) was applied to the DESeqDataSet prepared from KO count table to reduce the heteroskedasticity and its subsequent effect in the downstream data analysis. Association of the gene population with treatments (Control and H57) was tested by Permutational Multivariate Analysis of Variance (PERMANOVA) of variance-stabilized data using adonis() function of the vegan package (Oksanen et al., 2016). Ordination of the samples was done by principal component analysis (PCA) of this data using rda() function in vegan (Oksanen et al., 2016).

Finally, the differential abundance of genes was calculated by calling the function DESeq(), which calculated differential abundance of genes between treatments through estimation of size factors, estimation of dispersion and fitting a generalized linear model. This analysis enabled production of a table showing the effect of H57 on the effect size (log2 fold change) in the functional genes, standard error and p-values. The result was visualised with "MA-plot", which is a scatter plot with the mean of normalized counts of genes on the x-axis and log2 fold changes on the y-axis.

Functional analysis of the microbial population against KEGG (Kanehisa, 2002) and the SEED (Overbeek et al., 2005) database at higher level broadly defined functional categories was undertaken in Rapid Annotations using Subsystems Technology of Metagenomics (MG-RAST) server (Meyer et al., 2008). Shotgun sequences were uploaded to MG-RAST server after merging pair ends and run in its sequence-processing pipeline for subsequent analysis. Shotgun sequences were compared with KEGG Orthology (KO) and SEED subsystem based approach (Overbeek et al., 2005) and annotated to level 1, level 2 and level 3 functional categories using default parameters (maximum e-value = 5, minimum identity = 60%, minimum alignment length = 15, minimum abundance = 1). Significance of difference in overall functional capacity at particular level is tested with PERMANOVA using vegan package in R after Hellinger transformation (Legendre and Gallagher, 2001) of normalised abundance data while differences in relative abundance of particular functional category between treatments was tested with t-test.

5.2.3.2 Taxonomic analysis

Taxonomic profiles of the samples were analysed by using community profiling tool GraftM (Boyd et al., 2015) which identifies 16S rRNA genes in metagenomic sequences and creates community composition. The output from GraftM is normalised by rarefying in QIIME (Caporaso et al., 2010) and adjusted for 16S rRNA gene copy number variances using CopyRighter (Angly et al., 2014).

Downstream analysis was done in R as described in chapter 4 to analyse OTU data. The effect of H57 on microbial community composition was analysed by PERMANOVA using the package vegan (Oksanen et al., 2016). PCA was undertaken after Hellinger transformation (Legendre and Gallagher, 2001) of OTU relative abundance data. The relative abundances of the OTUs between treatments were also compared with a heatmap using the packages ggplot2 (Wickham, 2016) and RColorBrewer (Neuwirth, 2014). Indicator species (Dufrene and Legendre, 1997) were identified by using the function Indval() of the R package labdsv. The composition of microbial communities in different treatments was visualised using Krona (Ondov et al., 2011).

5.3 Results

5.3.1 Gene centric analysis

5.3.1.1 Run statistics

An average of 4.9 million sequences were obtained per sample (st. dev. ≈1.08 million) (Table 5-1). KEGG Orthology (KO) identifiers were assigned to 8,628 molecular level functions.

Table 5-1 Number of base pairs and sequence reads per sample

Sample ID	bp count	No. of reads	Treatment	Average reads per sample	SD
SA1672	1381122671	4978846	Sorghum_Control	5,345,929	602,869
SA1673	1741923983	6380251			
SA1674	1462595220	5339668			
SA1675	1343509175	4891447			
SA1676	1405727028	5139432			
SA1677	1542344800	5574657	Sorghum_H57	6,071,193	1,117,517
SA1679	1612437500	6095116			
SA1680	1465262776	5426908			
SA1681	2206585988	7991520			
SA1682	1445061318	5267763			
SA1695	1149773851	4367649	Wheat_Control	4,108,906	653,424
SA1696	1262686589	4818896			
SA1697	1161297440	4388486			
SA1698	1024948122	3858590			
SA1699	821343406	3110910			
SA1701	1185320663	4588689	Wheat_H57	4,143,848	285,562
SA1702	1030360374	3864356			
SA1703	1046709699	4030524			
SA1704	1124210788	4251978			
SA1705	1040037766	3983692			

5.3.1.2 Influence of H57 on KO profiles in sorghum and wheat-fed chickens

PERMANOVA indicated that there was significant difference in the molecular-level functions (KOs associated with microbial communities) between the Control and H57 treatments for sorghum ($p < 0.01$) and wheat ($p < 0.05$) diets. Therefore, H57 was associated with significant change in the molecular level functional capacity of the microbiome in both diets. PCA also showed the moderate segregation of Control and H57 treated birds both in sorghum (**Figure 5-1**) and wheat (Figure 5-2) based diets.

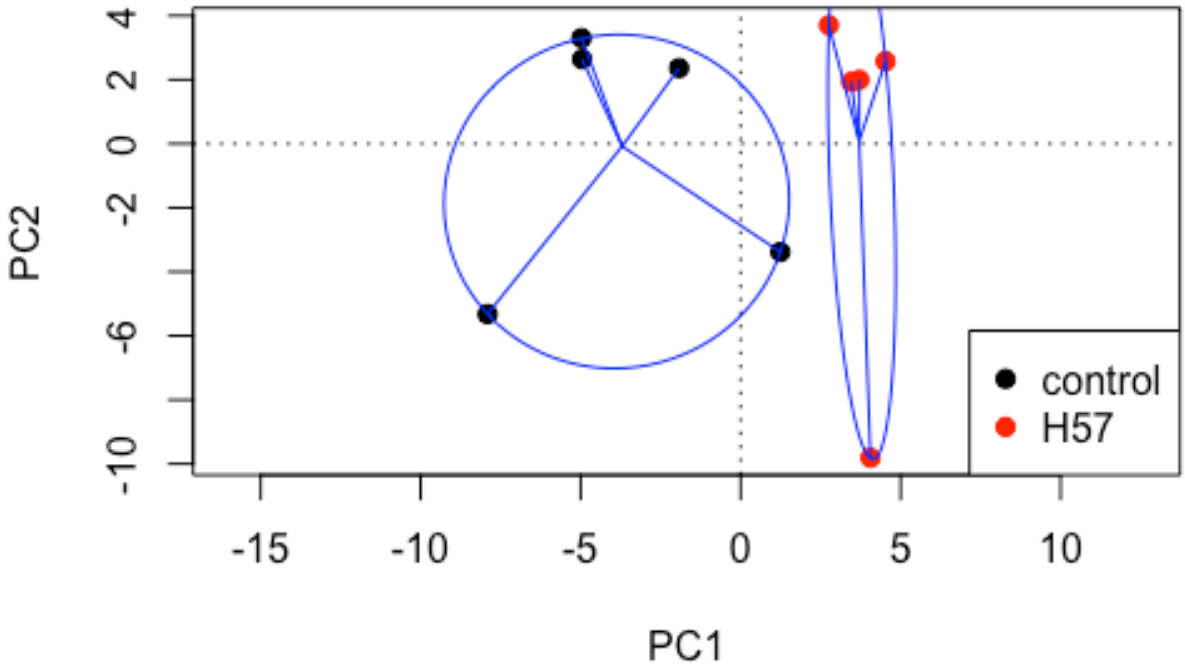


Figure 5-1 Principal component analysis of variance-stabilizing transformed KO function matrix for sorghum based diet.

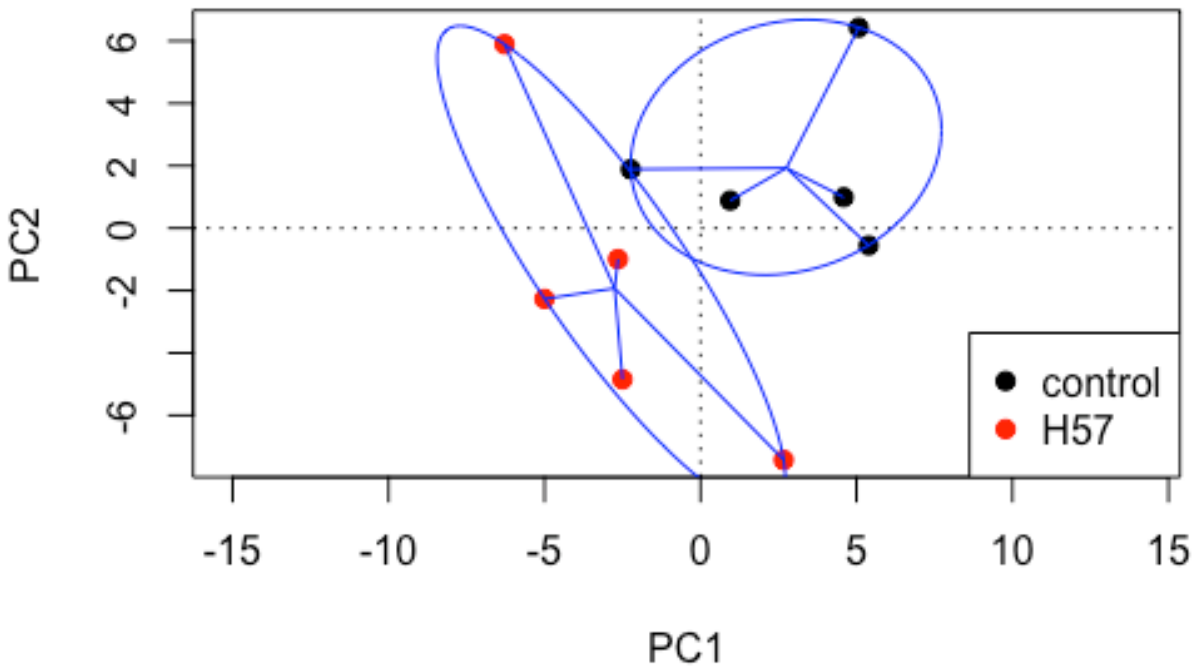


Figure 5-2 Principal component analysis of variance-stabilizing transformed KO function matrix for wheat based diet.

The number of genes affected by H57 was greater in sorghum-fed birds than in wheat-fed birds. In addition, the effect size (log2 fold change) of H57 on the affected genes was larger in sorghum-fed birds than in wheat-fed birds. Genes with a log2 fold change value of at least ±5 between Control and H57 for sorghum-based diet are given in Table 5-2 and genes with a log2 fold change value of at least ±2 between Control and H57 for wheat based diet are presented in Table 5-3. The MA-plots showing gene wise dispersion over the mean of counts normalized by size factor are shown in Figure 5-3 (sorghum) and Figure 5-4 (wheat).

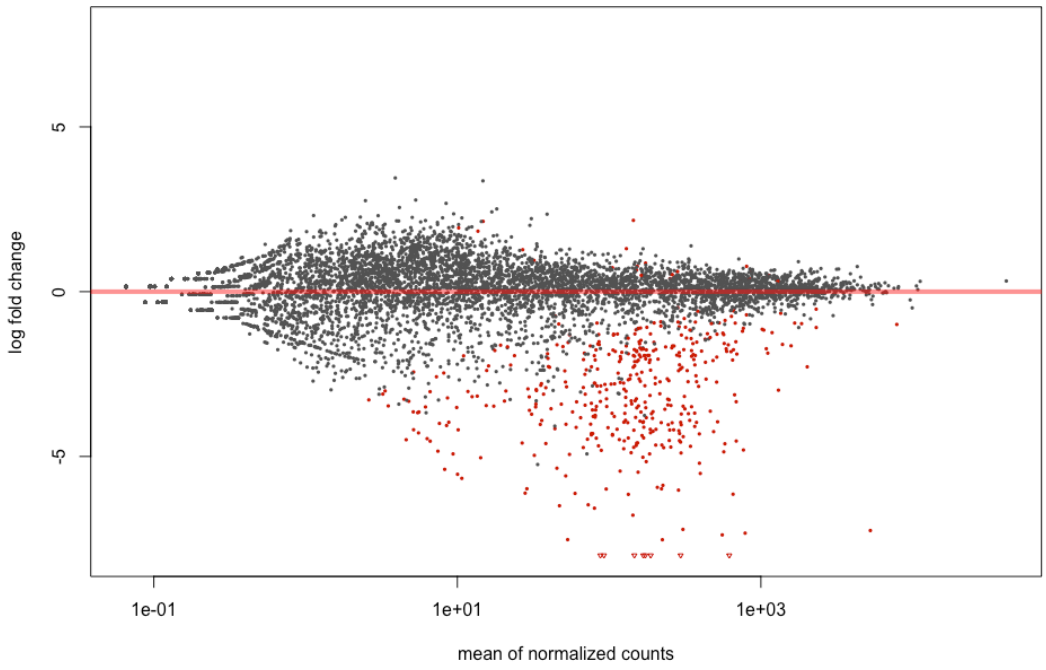


Figure 5-3 MA-plot (sorghum) shows the gene wise dispersion (log2 fold change) over the mean between Control and H57 in chickens fed sorghum based diet. Red = significantly different. Positive fold change is higher abundance of genes in H57 and negative fold change is higher abundance in Control birds. It is evident from the plot that there are several genes represented that are higher in Control than in H57 as demonstrated by more red dots on the negative axis sides.

Table 5-2 Genes in caeca with significant differential abundance between Control and H57 birds fed sorghum based diet with log2 fold change value of at least 5. Positive fold change indicates higher abundance in H57 groups and negative fold change indicates higher abundance in Control group. Padj is Benjamini-Hochberg (BH) adjusted (Benjamini and Hochberg, 1995) p-value.

ID	log2FoldChange	pvalue	padj	annotations
K01277	-9.8	2.2E-24	3.8E-21	DPP3; dipeptidyl-peptidase III [EC:3.4.14.4]
K08676	-9.5	1.2E-34	6.0E-31	tri; tricorn protease [EC:3.4.21.-]
K07405	-9.0	4.7E-19	2.2E-16	E3.2.1.1A; alpha-amylase [EC:3.2.1.1]
K13043	-8.9	4.9E-19	2.2E-16	argF; N-succinyl-L-ornithine transcarbamylase [EC:2.1.3.11]
K12343	-8.5	1.7E-17	5.6E-15	SRD5A1; 3-oxo-5-alpha-steroid 4-dehydrogenase 1 [EC:1.3.1.22]
K00346	-8.5	2.1E-19	1.4E-16	nqrA; Na+-transporting NADH:ubiquinone oxidoreductase subunit A [EC:1.6.5.8]
K00210	-8.4	2.6E-17	8.3E-15	E1.3.1.12; prephenate dehydrogenase [EC:1.3.1.12]
K01173	-8.1	1.8E-18	7.1E-16	ENDOG; endonuclease G, mitochondrial
K09789	-7.7	2.0E-13	4.4E-11	bioG; pimeloyl-[acyl-carrier protein] methyl ester esterase [EC:3.1.1.85]
K09011	-7.5	3.4E-24	4.2E-21	cimA; D-citramalate synthase [EC:2.3.1.182]
K02429	-7.4	3.4E-25	8.6E-22	fucP; MFS transporter, FHS family, L-fucose permease
K01278	-7.3	2.2E-19	1.4E-16	DPP4, CD26; dipeptidyl-peptidase 4 [EC:3.4.14.5]
K02014	-7.2	3.0E-19	1.7E-16	TC.FEV.OM; iron complex outermembrane recepter protein
K03832	-7.2	6.9E-21	6.9E-18	tonB; periplasmic protein TonB

K03444	-7.0	4.0E-10	4.2E-08	MFS.SP; MFS transporter, SP family, sugar porter, other
K14440	-6.8	8.7E-14	2.1E-11	SMARCAL1, HARP; SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A-like protein 1 [EC:3.6.4.12]
K00349	-6.6	1.1E-13	2.4E-11	nqrD; Na+-transporting NADH:ubiquinone oxidoreductase subunit D [EC:1.6.5.8]
K15789	-6.5	4.2E-10	4.3E-08	TDH; threonine 3-dehydrogenase [EC:1.1.1.103]
K15727	-6.5	7.1E-12	1.0E-09	czcB; membrane fusion protein, cobalt-zinc-cadmium efflux system
K10742	-6.1	3.7E-13	7.7E-11	DNA2; DNA replication ATP-dependent helicase Dna2 [EC:3.6.4.12]
K01284	-6.1	3.6E-11	4.8E-09	dcp; peptidyl-dipeptidase Dcp [EC:3.4.15.5]
K15255	-6.1	7.7E-12	1.1E-09	PIF1; ATP-dependent DNA helicase PIF1 [EC:3.6.4.12]
K17744	-6.1	5.1E-13	9.8E-11	GalDH; L-galactose dehydrogenase [EC:1.1.1.316]
K03561	-6.0	5.9E-20	4.9E-17	exbB; biopolymer transport protein ExbB
K07164	-6.0	2.8E-15	8.2E-13	K07164; uncharacterized protein
K06142	-6.0	1.5E-18	6.3E-16	hlpA, ompH; outer membrane protein
K18303	-6.0	6.2E-11	7.6E-09	mexK; multidrug efflux pump
K10532	-6.0	6.4E-08	3.0E-06	HGSNAT; heparan-alpha-glucosaminide N-acetyltransferase [EC:2.3.1.78]
K16363	-5.9	5.3E-18	1.9E-15	lpxC-fabZ; UDP-3-O-[3-hydroxymyristoyl] N-acetylglucosamine deacetylase / 3-hydroxyacyl-[acyl-carrier-protein] dehydratase [EC:3.5.1.108 4.2.1.59]
K16053	-5.9	3.1E-14	8.5E-12	mscM; miniconductance mechanosensitive channel
K01078	-5.9	1.4E-07	6.1E-06	E3.1.3.2; acid phosphatase [EC:3.1.3.2]
K09516	-5.7	2.5E-07	9.9E-06	RETSAT; all-trans-retinol 13,14-reductase [EC:1.3.99.23]
K03113	-5.6	1.7E-12	2.8E-10	EIF1, SUI1; translation initiation factor 1
K12340	-5.5	6.5E-14	1.6E-11	tolC; outer membrane protein
K19049	-5.5	2.1E-10	2.3E-08	cslA; chondroitin AC lyase [EC:4.2.2.5]
K11537	-5.4	1.2E-10	1.5E-08	xapB; MFS transporter, NHS family, xanthosine permease
K00889	-5.3	4.5E-09	3.2E-07	PIP5K; 1-phosphatidylinositol-4-phosphate 5-kinase [EC:2.7.1.68]
K16327	-5.3	7.0E-06	1.9E-04	K16327; putative LysE/RhtB family amino acid efflux pump
K09650	-5.2	8.7E-06	2.3E-04	PARL, PSARL, PCP1; rhomboid-like protein [EC:3.4.21.105]
K15726	-5.2	5.6E-10	5.5E-08	czcA; cobalt-zinc-cadmium resistance protein CzcA
K08289	-5.1	2.5E-08	1.3E-06	purT; phosphoribosylglycinamide formyltransferase 2 [EC:2.1.2.2]
K18299	-5.0	1.9E-07	7.8E-06	mexF; multidrug efflux pump
K08138	-5.0	8.5E-08	3.9E-06	xylE; MFS transporter, SP family, xylose:H+ symportor

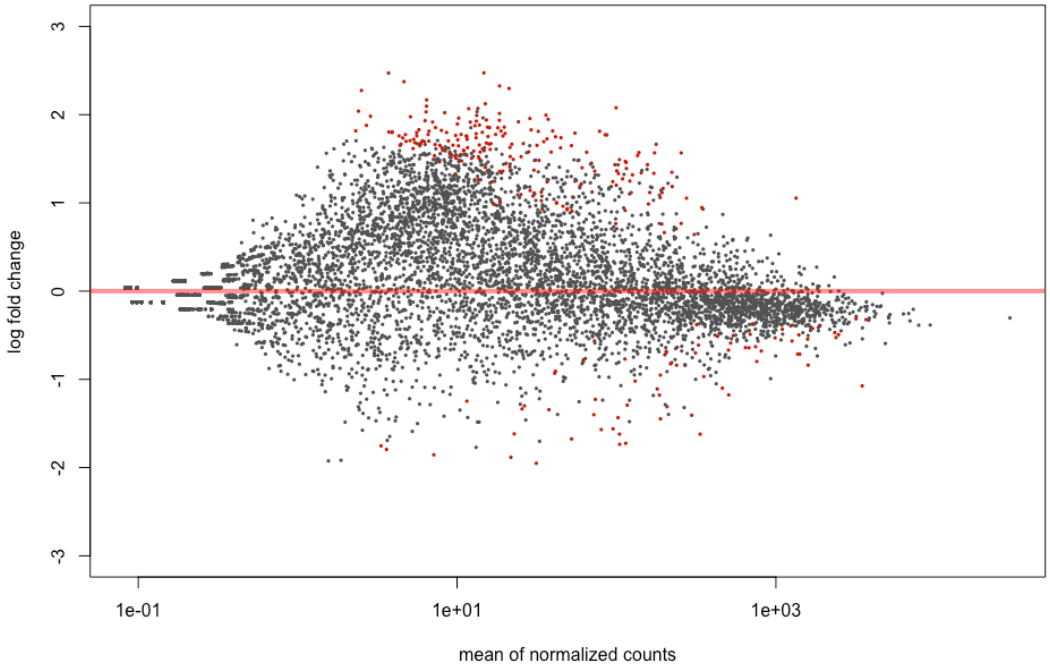


Figure 5-4 MA-plot (wheat) shows the gene wise dispersion (log2 fold change) over the mean between Control and H57 in chickens fed wheat based diet. Red = significantly different. Positive fold change is higher in H57 and negative fold change in higher in Control birds. In contrast to sorghum based diet, several genes are representatively higher in H57 treated birds than in Control birds groups.

Table 5-3 Genes in caeca with significant differential abundance between Control and H57 birds fed wheat based diet with log2 fold change (estimate of effect size) value of at least 2. Positive fold change (green colour cells) indicates higher in H57 groups and negative fold change (orange color cells) indicates higher in Control group. Padj is Benjamini-Hochberg (BH) adjusted (Benjamini and Hochberg, 1995) p-value.

ID	log2 fold change	pvalue	padj	annotations
K02344	2.6	0.000047	0.01	DPO3P, holD; DNA polymerase III subunit psi [EC:2.7.7.7]
K00844	2.5	0.000087	0.01	HK; hexokinase [EC:2.7.1.1]
K00389	2.4	0.000052	0.01	yidH; putative membrane protein
K03543	2.3	0.000003	0.00	emrA; membrane fusion protein, multidrug efflux system
K07798	2.3	0.000015	0.01	cusB, silB; membrane fusion protein, Cu(I)/Ag(I) efflux system
K15829	2.2	0.000199	0.02	hycD; formate hydrogenlyase subunit 4
K16091	2.2	0.000704	0.04	fecA; Fe(3+) dicitrate transport protein
K11391	2.1	0.000558	0.03	rlmG; 23S rRNA (guanine1835-N2)-methyltransferase [EC:2.1.1.174]
K04091	2.1	0.000260	0.03	ssuD; alkanesulfonate monooxygenase [EC:1.14.14.5]
K10906	2.1	0.000093	0.01	recE; exodeoxyribonuclease VIII [EC:3.1.11.-]
K06894	2.1	0.000005	0.00	K06894; uncharacterized protein
K07008	2.1	0.000446	0.03	egtC; gamma-glutamyl hercynylcysteine S-oxide hydrolase [EC:3.5.1.118]
K11735	2.0	0.000308	0.03	gabP; GABA permease
K06144	2.0	0.001183	0.05	uspB; universal stress protein B
K02336	2.0	0.000484	0.03	DPO2, polB; DNA polymerase II [EC:2.7.7.7]

K08137	2.0	0.000651	0.04	galP; MFS transporter, SP family, galactose:H+ symporter
K00983	2.0	0.000415	0.03	E2.7.7.43, neuA, CMAS; N-acylneuraminate cytidylyltransferase [EC:2.7.7.43]
K15723	2.0	0.000514	0.03	syd; SecY interacting protein Syd
K13775	-2.1	0.001016	0.04	atuG; citronellol/citronellal dehydrogenase

Genes were classified into six KO categories at broadest level (KO level 1) of functional classification (Table 5-4), into 42 categories at KO function level 2 (Table 5-5) and into 156 categories at KO functional level 3 (details in appendix 3). These numbers represent the microbial functional diversity in the caeca of chickens at different level of classification. Although there was significant difference between Control and H57 in overall microbial functional capacity at molecular-functional level for both sorghum and wheat based diet as described above, there was no significant difference in potential function at KO level 1 (P = 0.16), level 2 (P = 0.95) and level 3 (P = 0.63) for sorghum-fed chickens. For wheat-fed chickens, functional capacity between Control and H57 was not different in KO level 1 (P = 0.46) whereas it was significantly different at KO level 2 (P = 0.045) and level 3 (P = 0.049).

Table 5-4 KO level 1 functional classification of the sequence reads with average relative abundance.

KO Level 1	**Percentage of reads (relative abundance)**					
	Sorghum			**Wheat**		
	Control	**H57**	**P-value**	**Control**	**H57**	**P-value**
Cellular Processes	3.48	2.99	0.11	3.28	2.67	0.04
Environmental Information Processing	11.66	14.53	0.10	14.66	14.87	0.82
Genetic Information Processing	25.23	26.11	0.74	24.44	24.81	0.87
Metabolism	57.79	55.13	0.30	56.44	56.05	0.86
Human Diseases	1.02	0.73	0.21	0.76	1.18	0.04
Organismal Systems	0.81	0.51	0.11	0.43	0.41	0.83

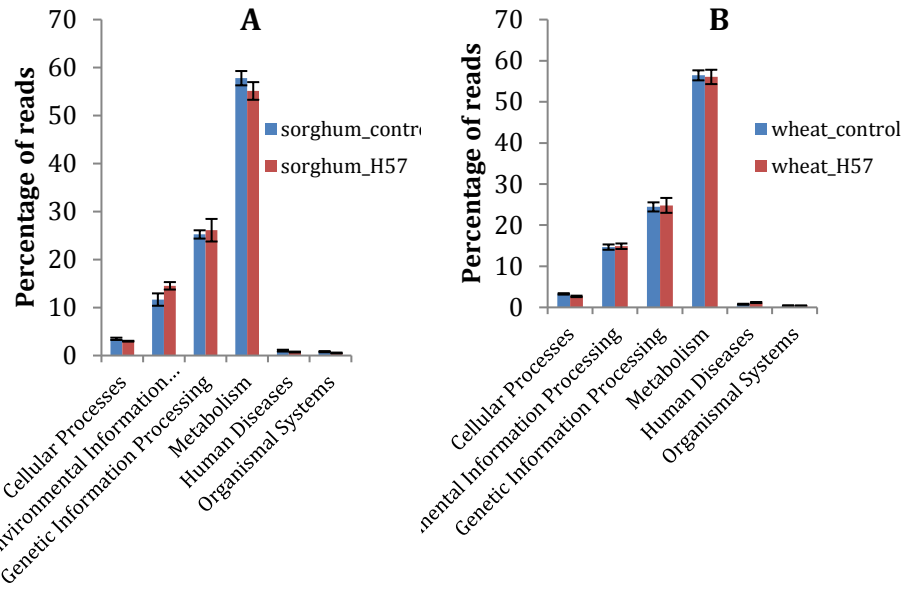


Figure 5-5 Comparison of KEGG Orthology (KO) Level 1 functions between Control and H57 groups A) Sorghum based diet B) Wheat based diet. Error bars are standard error of mean (SEM).

There were several individual functional pathways different at different levels of significance between Control and H57. At KO level 1, relative abundance (percentage of reads) of genes for cellular process was significantly lower (P = 0.04) in H57 groups for wheat based diet (Figure 5-5). There was no difference between Control and H57 groups at KO functional level 2 (Table 5-5, Figure 5-6 and Figure 5-7) both in the sorghum and wheat based diets. However, at KO functional level 3, a number of pathways had significantly different relative abundance between Control and H57 birds (Table 5-6). For sorghum-based diet, genes responsible for Tricarboxylic Acid Cycle (TCA cycle) (P = 0.03), Glutathione metabolism (P = 0.03), vitamin B6 metabolism (P = 0.01), pantothenate and CoA biosynthesis (P = 0.02), and protein processing in endoplasmic reticulum (P = 0.02) had significantly lower relative abundance in H57 treated groups while genes for carbon fixation pathways in prokaryotes (P = 0.03), and phosphotransferase system (P = 0.01) had significantly higher relative abundance in the H57 group when compared with birds on the Control diet (**Figure 5-8**). Similarly, for the wheat-based diet, genes for propanoate metabolism (P = 0.01) and thiamine metabolism (P = 0.03) had significantly lower relative abundance in the H57 group while genes responsible for the TCA cycle (P = 0.03), pentose phosphate pathway (P = 0.03), and riboflavin metabolism (P = 0.03) were represented at significantly lower in H57 treated birds when compared with the Control group (Figure 5-9).

Table 5-5 KO level 2 functional classification of the sequence reads with average relative abundance.

KO level 2	Percentage of reads					
	Sorghum			Wheat		
	Control	H57	P-value	Control	H57	P-value
Cell communication	20.18	20.52	0.761	20.67	19.16	0.445
Cell growth and death	16.54	17.25	0.210	16.12	16.78	0.544
Cell motility	10.93	11.58	0.621	10.58	10.81	0.845
Transport and catabolism	9.10	12.25	0.096	12.07	12.40	0.658
Membrane transport	6.41	6.64	0.744	6.23	5.77	0.610
Signal transduction	5.63	5.96	0.339	5.95	5.49	0.175
Signalling molecules and interaction	4.37	2.75	0.054	3.29	3.33	0.816
Folding, sorting and degradation	4.17	4.45	0.652	4.22	4.05	0.832
Replication and repair	3.72	3.45	0.330	3.40	3.64	0.342
Transcription	3.45	3.11	0.402	3.68	3.65	0.610
Translation	2.56	2.28	0.278	2.59	2.43	0.569
Cancers	2.29	2.33	0.872	2.48	1.87	0.104
Endocrine and metabolic diseases	2.04	2.08	0.831	2.09	2.55	0.284
Immune diseases	1.99	1.02	0.091	1.21	1.19	0.934
Infectious diseases	1.97	1.32	0.253	1.73	2.13	0.177
Neurodegenerative diseases	1.11	0.78	0.072	1.12	0.69	0.069
Substance dependence	0.88	0.29	0.107	0.42	0.41	0.942
Amino acid metabolism	0.68	0.33	0.133	0.51	0.84	0.067
Biosynthesis of other secondary metabolites	0.55	0.51	0.755	0.37	0.38	0.880
Carbohydrate metabolism	0.41	0.27	0.276	0.53	0.65	0.503
Energy metabolism	0.31	0.39	0.069	0.25	0.28	0.809
Glycan biosynthesis and metabolism	0.31	0.37	0.566	0.38	0.43	0.720
Lipid metabolism	0.24	0	0.105	0.05	0.07	0.803
Metabolism of cofactors and vitamins	0.10	0.06	0.415	0.04	0.08	0.116
Metabolism of other amino acids	0.03	0	0.296			
Metabolism of terpenoids and polyketides	0.02	0	0.235	0.01	0	0.301
Xenobiotics biodegradation and metabolism	0	0.01	0.253	0	0.02	0.232

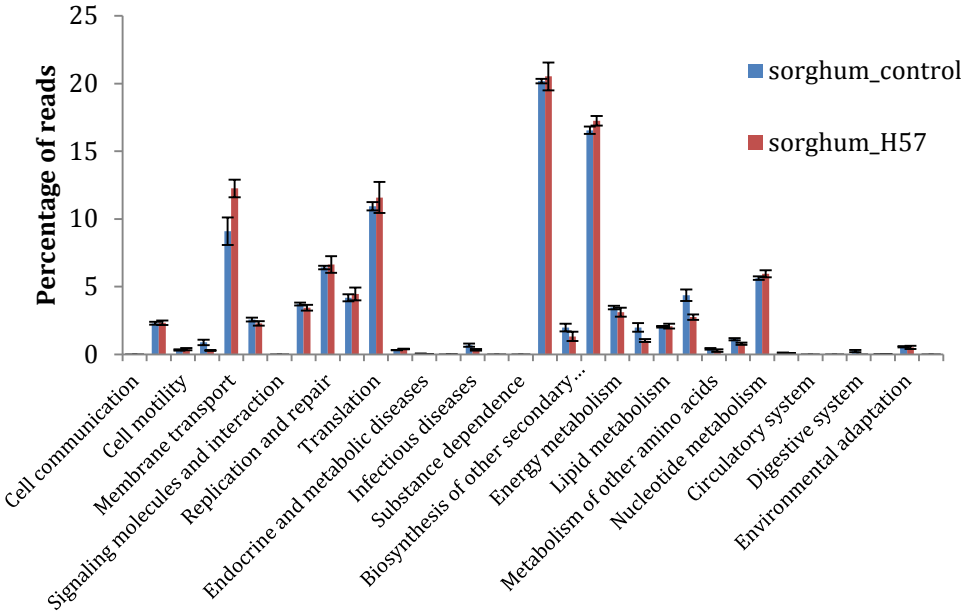


Figure 5-6 Comparison of KEGG Orthology (KO) Level 2 functions between Control and H57 birds fed sorghum based diet. Error bars are SEM.

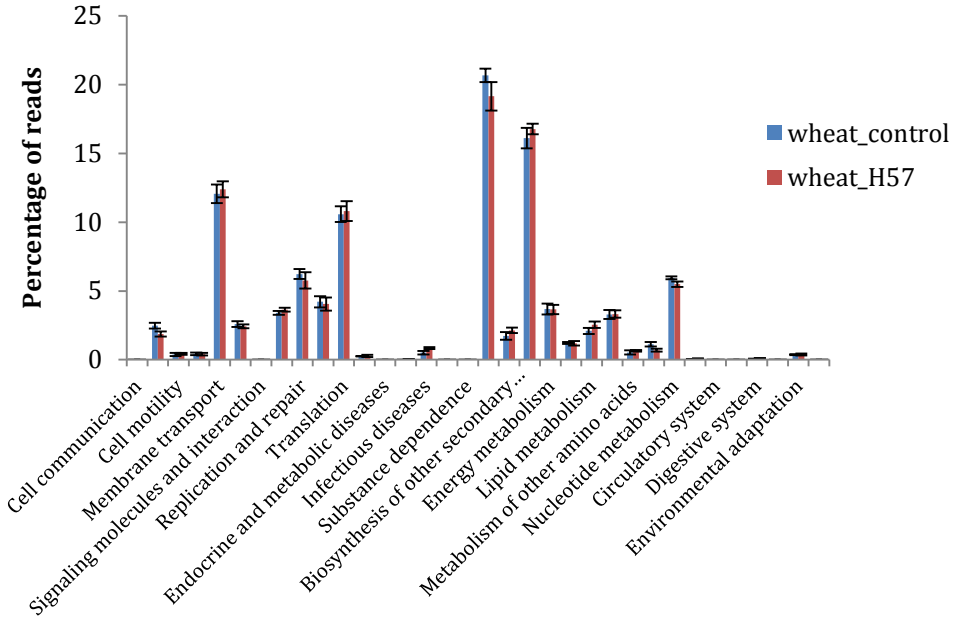


Figure 5-7 Comparison of KEGG Orthology (KO) Level 2 functions between Control and H57 birds fed wheat based diet. Error bars are SEM.

Table 5-6 KO level 3 functional pathways with significantly different relative abundance (% of total reads) between Control and H57. Relative abundances are average of five replicate chickens.

Sorghum				Wheat			
KO level 3 pathway	Percentage of reads			KO level 3 pathway	Percentage of reads		
	Control	H57	P-value		Control	H57	P-value
00020 Citrate cycle (TCA cycle) [PATH:ko00020]	1.08	0.51	0.03	00020 Citrate cycle (TCA cycle) [PATH:ko00020]	0.59	0.84	0.03
00480 Glutathione metabolism [PATH:ko00480]	0.22	0.07	0.03	00030 Pentose phosphate pathway [PATH:ko00030]	1.36	1.83	0.03
00720 Carbon fixation pathways in prokaryotes [PATH:ko00720]	0.37	0.43	0.04	00640 Propanoate metabolism [PATH:ko00640]	0.06	0.01	0.01
00750 Vitamin B6 metabolism [PATH:ko00750]	0.25	0.03	0.01	00730 Thiamine metabolism [PATH:ko00730]	0.60	0.45	0.03
00770 Pantothenate and CoA biosynthesis [PATH:ko00770]	0.43	0.27	0.02	00740 Riboflavin metabolism [PATH:ko00740]	0.09	0.21	0.03
02060 Phosphotransferase system (PTS) [PATH:ko02060]	0.78	1.51	0.01				
04141 Protein processing in endoplasmic reticulum [PATH:ko04141]	0.02	0.00	0.02				

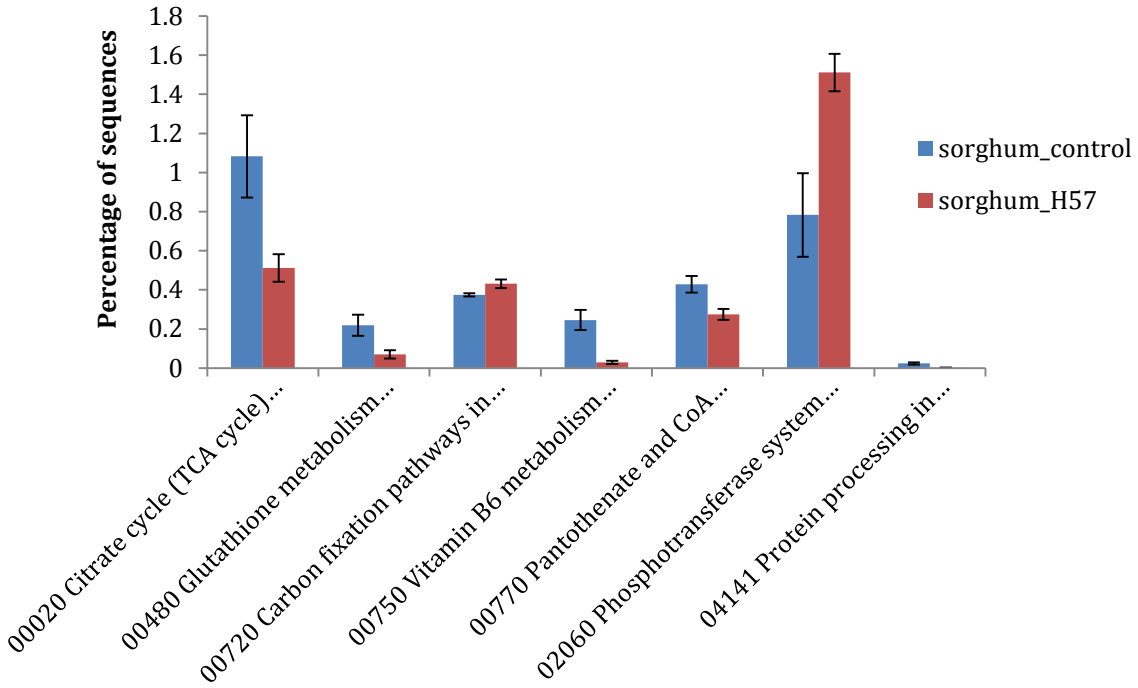


Figure 5-8 Comparison of KEGG Orthology (KO) Level 3 functions which were significantly different between Control and H57 birds fed sorghum based diet. Error bars are SEM

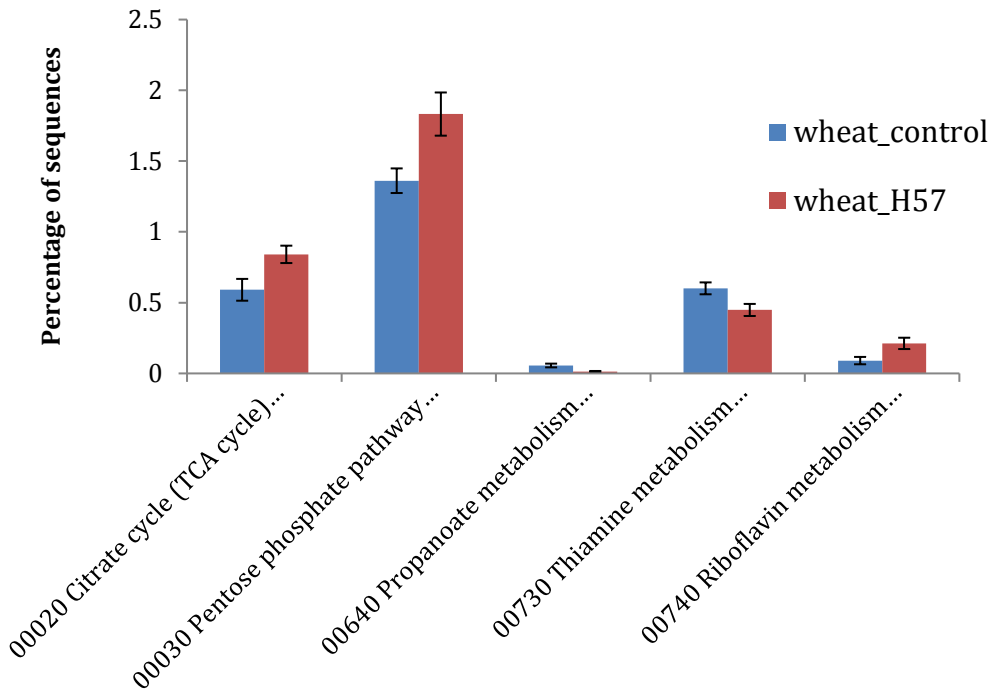


Figure 5-9 Comparison of KEGG Orthology (KO) Level 3 functions which were significantly different between Control and H57 birds fed wheat based diet. Error bars are SEM.

5.3.1.3 SEED Subsystem

Annotation and classification of genomes according SEED subsystem classified genes into 28 functional categories at subsystem level 1, into 154 categories at level 2 and into 953 categories at level 3 indicating the caecal microbial functional diversity. For wheat-fed birds, there was no significant difference for overall functional capacity between Control and H57 at level 1 (P = 0.10), level 2 (P = 0.57) and level 3 (P = 0.40). Although there was no difference at the broadest level of functional classification (subsystem level 1) between Control and H57 for sorghum-fed birds, the differences were significant at level 2 (P = 0.03) and level 3 (P = 0.03).

At subsystem level 1, five subsystems were significantly different between the Control and H57 group in the sorghum-based diet (Table 5-1). Relative abundance of genes classified as “cell division and cell cycle” (P = 0.003) and “stress response” (P = 0.04) were significantly higher in the birds fed H57 as compared to birds fed the Control diet while genes responsible for “cell wall and capsule” (P = 0.01) and “potassium metabolism” (P = 0.04) had significantly lower relative abundance in the H57 group than in Control birds (Figure 5-10 and Figure 5-11). However, there was no difference in subsystems level 1 in the samples collected from the birds fed wheat based diet (Table 5-7 and Figure 5-11). Seventeen different functional groups, as presented in Table 5-8 and Figure 5-12, had significantly different relative abundance between the Control and H57 groups for the sorghum-based diet. Only six functional groups at subsystem level 2 had significantly different

relative abundance between the Control and H57 groups for the wheat-based diet (Table 5-9). A large proportion (about one fifth) of the sequences were unclassified from the wheat based diet both in the Control and H57 groups.

Table 5-7 SEED subsystem level 1 functional classification of the sequence reads with average relative abundance. Numbers in bold are significantly different between Control and H57.

Subsystem level 1	Relative abundance (percentage of the reads)					
	Sorghum			Wheat		
	Control	H57	P-value	Control	H57	P-value
Amino Acids and Derivatives	8.88	8.75	0.89	9.68	9.61	0.93
Carbohydrates	17.56	18.73	0.34	17.39	17.65	0.85
Cell Division and Cell Cycle	**1.39**	**2.14**	**0.00**	1.85	1.83	0.94
Cell Wall and Capsule	**4.07**	**2.67**	**0.01**	3.54	3.33	0.57
Clustering-based subsystems	12.34	13.15	0.51	13.42	12.70	0.57
Cofactors, Vitamins, Prosthetic Groups, Pigments	3.78	2.98	0.05	3.32	3.27	0.91
DNA Metabolism	6.93	7.81	0.14	7.05	6.43	0.31
Dormancy and Sporulation	0.40	0.79	0.06	0.53	0.44	0.58
Fatty Acids, Lipids, and Isoprenoids	1.47	1.48	0.92	1.76	1.94	0.47
Iron acquisition and metabolism	1.12	0.80	0.09	0.86	0.50	0.19
Membrane Transport	2.86	3.30	0.27	3.29	3.53	0.46
Metabolism of Aromatic Compounds	0.31	0.44	0.23	0.32	0.44	0.18
Miscellaneous	4.66	4.41	0.42	5.05	5.15	0.84
Motility and Chemotaxis	0.15	0.14	0.90	0.10	0.14	0.21
Nitrogen Metabolism	0.71	0.78	0.67	0.69	0.61	0.68
Nucleosides and Nucleotides	3.50	3.80	0.28	3.99	4.20	0.69
Phages, Prophages, Transposable elements, Plasmids	4.54	3.20	0.10	3.11	3.06	0.95
Phosphorus Metabolism	1.22	1.03	0.22	1.39	1.09	0.24
Photosynthesis	0.03	0.00	0.12	0.00	0.01	0.49
Potassium metabolism	**0.39**	**0.14**	**0.04**	0.40	0.34	0.66
Protein Metabolism	9.98	10.04	0.95	9.76	10.61	0.33
RNA Metabolism	5.14	5.18	0.92	5.10	5.54	0.32
Regulation and Cell signalling	0.80	0.61	0.05	0.64	0.78	0.44
Respiration	2.86	2.97	0.84	2.84	3.29	0.62
Secondary Metabolism	0.03	0.09	0.14	0.09	0.10	0.86
Stress Response	**1.22**	**1.62**	**0.04**	1.18	1.25	0.68
Sulfur Metabolism	0.62	0.57	0.66	0.68	0.38	0.09
Virulence, Disease and Defence	3.04	2.36	0.06	1.94	1.77	0.59

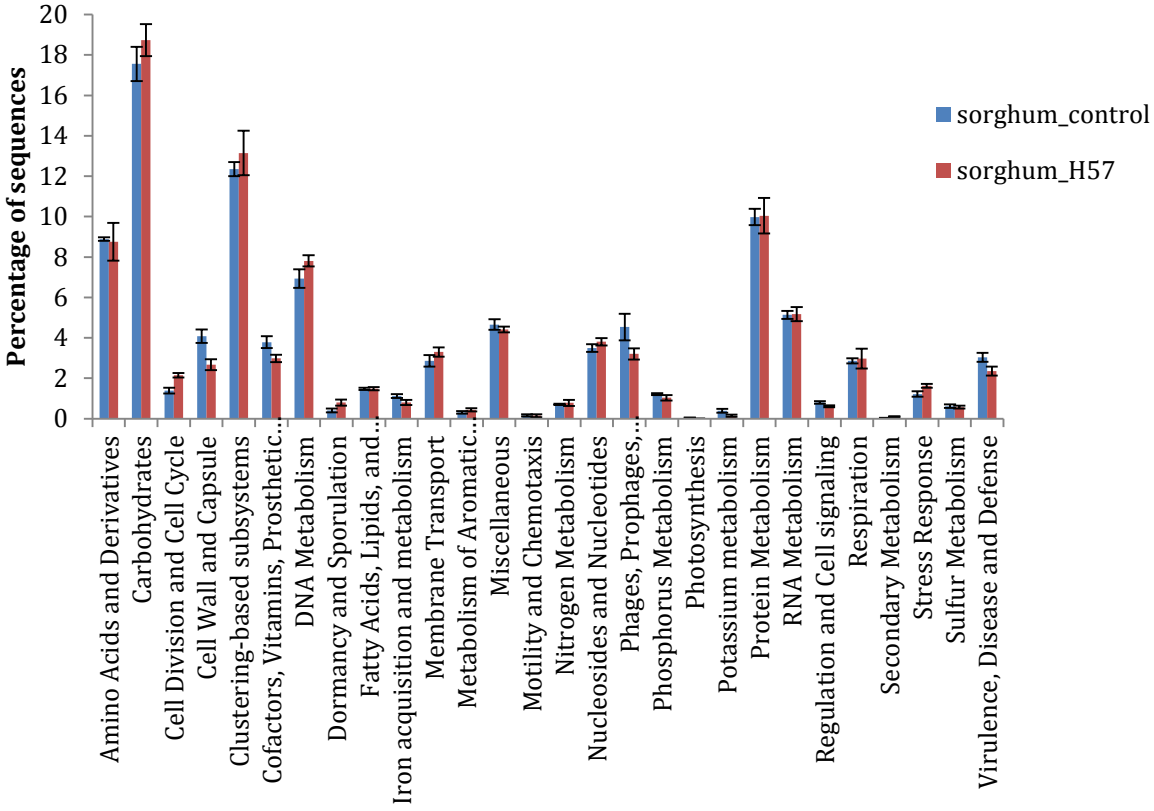


Figure 5-10 Functional analysis based on Subsystems level 1 classification in the caeca of birds fed sorghum based diet. Error bars are SEM.

Table 5-8 Subsystems level 2 functions significantly different between H57 and Control birds fed sorghum based diet.

Subsystem level 2	Percentage of sequences		P-value
	Control	H57	
Capsular and extracellular polysacchrides	1.81	1.28	0.027
Carbohydrates	0.05	0.13	0.014
Clustering-based subsystems	0.39	0.57	0.011
DNA uptake, competence	0.25	0.48	0.033
Gram-Negative cell wall components	0.77	0.18	0.026
Heat shock	0.42	0.64	0.014
One-carbon Metabolism	1.10	0.60	0.028
Probably GTP or GMP signaling related	0.19	0.05	0.006
Putative GGDEF domain protein related to agglutinin secretion	0.06	0.00	0.042
Pyridoxine	0.32	0.24	0.007
Pyruvate kinase associated cluster	0.01	0.11	0.019
Regulation of virulence	0.22	0.07	0.047
Resistance to antibiotics and toxic compounds	2.56	1.33	0.006
Reverse electron transport	0.11	0.31	0.047
Shiga toxin cluster	0.04	0.01	0.022
Spore DNA protection	0.04	0.09	0.016
TldD cluster	0.07	0.01	0.047

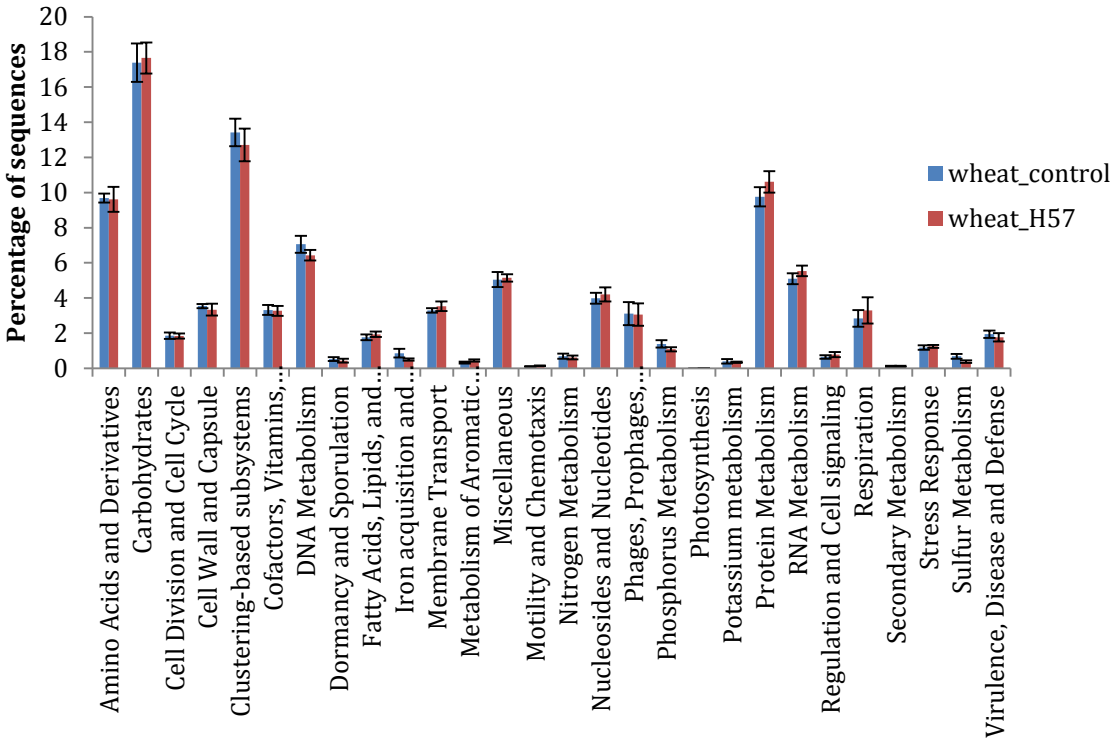


Figure 5-11 Functional analysis based on Subsystems level 1 classification in the caeca of birds fed wheat based diet. Error bars are SEM.

Table 5-9 Subsystems level 2 functions significantly different between H57 and Control birds fed wheat based diet.

Subsystems level 2	Percentage of sequences		P-value
	Control	H57	
Fermentation	1.661	2.144	0.03
Gram-Positive cell wall components	0.140	0.355	0.02
Hypothetical in Lysine biosynthetic cluster	0.170	0.046	0.02
Periplasmic Stress	0.001	0.012	0.01
Polysaccharides	0.710	0.431	0.03
Protein secretion system, Type VII (Chaperone/Usher pathway, CU)	0.003	0.016	0.03

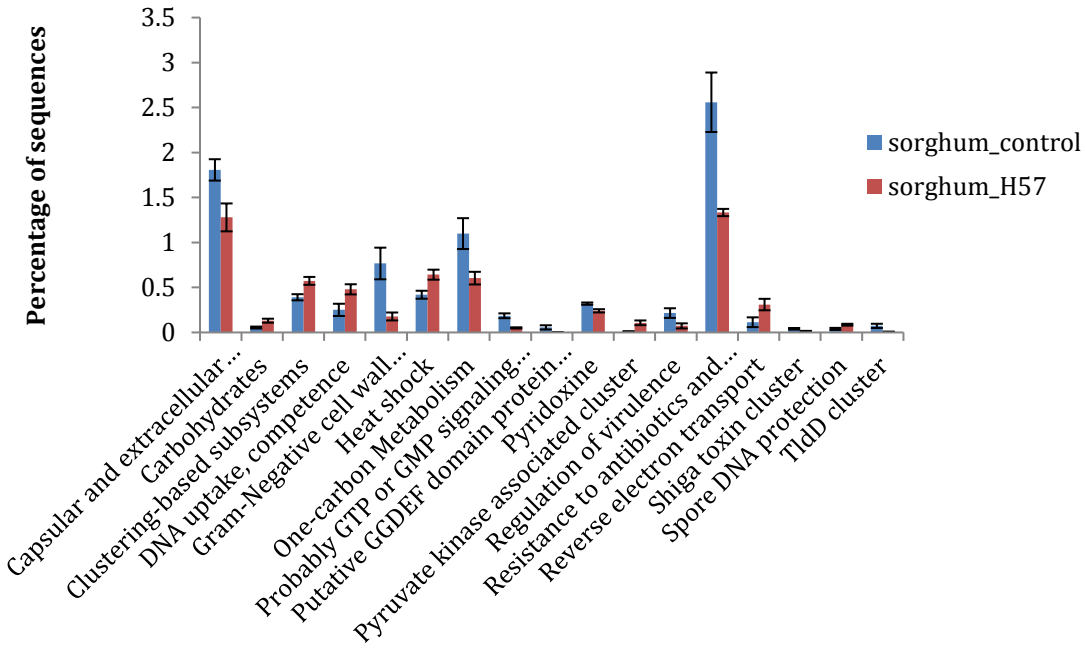


Figure 5-12 Functional analysis based on Subsystems level 2 classification in the caeca of birds fed sorghum based diet. Only subsystems which are significantly different between Control and H57 birds are presented. Error bars are SEM.

At subsystem level 3, sequences were classified into 953 functional groups. Out of these, 71 functional groups (complete list in appendix 3) were significantly more abundant for the H57-sorghum-based diet than in the Control birds. Similarly, 34 functional groups (complete list in appendix 3) had significantly different relative abundance between Control and H57 groups for the wheat-based diet. Only six functional groups were common between sorghum-fed birds and wheat-fed birds which were significantly different between Control and H57 groups indicating diet independent effect of H57 on microbial function.

5.3.1.4 Dominant microbial functions

Metabolism was the most prevalent functional class, representing ~56% of genes (~58% in Sorghum Control, 55% in Sorghum H57, 56% in Wheat Control and 56% in Wheat H57) followed by genetic information processing, representing ~25% of genes (~25% in Sorghum Control, 26% in Sorghum H57, 24% in Wheat Control and 25% in Wheat H57) at the KO level 1. Environmental information processing was the third most abundant KO level 1 category in all treatments representing ~15% except in Sorghum Control with ~12% relative abundance.

For the SEED subsystem based annotation, the top three functional gene categories in all treatment groups were genes related to carbohydrate utilisation (~18% in Sorghum Control, 19% in Sorghum H57, 17% in Wheat Control and 18% in Wheat H57), clustering-based subsystems (genes with evidence of functional coupling) (~12% in Sorghum Control, 13% in Sorghum H57, 13% in Wheat

Control and 13% in Wheat H57) and protein metabolism (~10% in Sorghum Control, 10% in Sorghum H57, 10% in Wheat Control and 11% in Wheat H57) (details in appendix 3). This conservation of microbial function also extended to dominant functional categories for more focussed classifications (KO levels 2 and 3 and SEED subsystems level 2) as revealed by similar relative abundance. (Figure 5.19 and Figure 5.20).

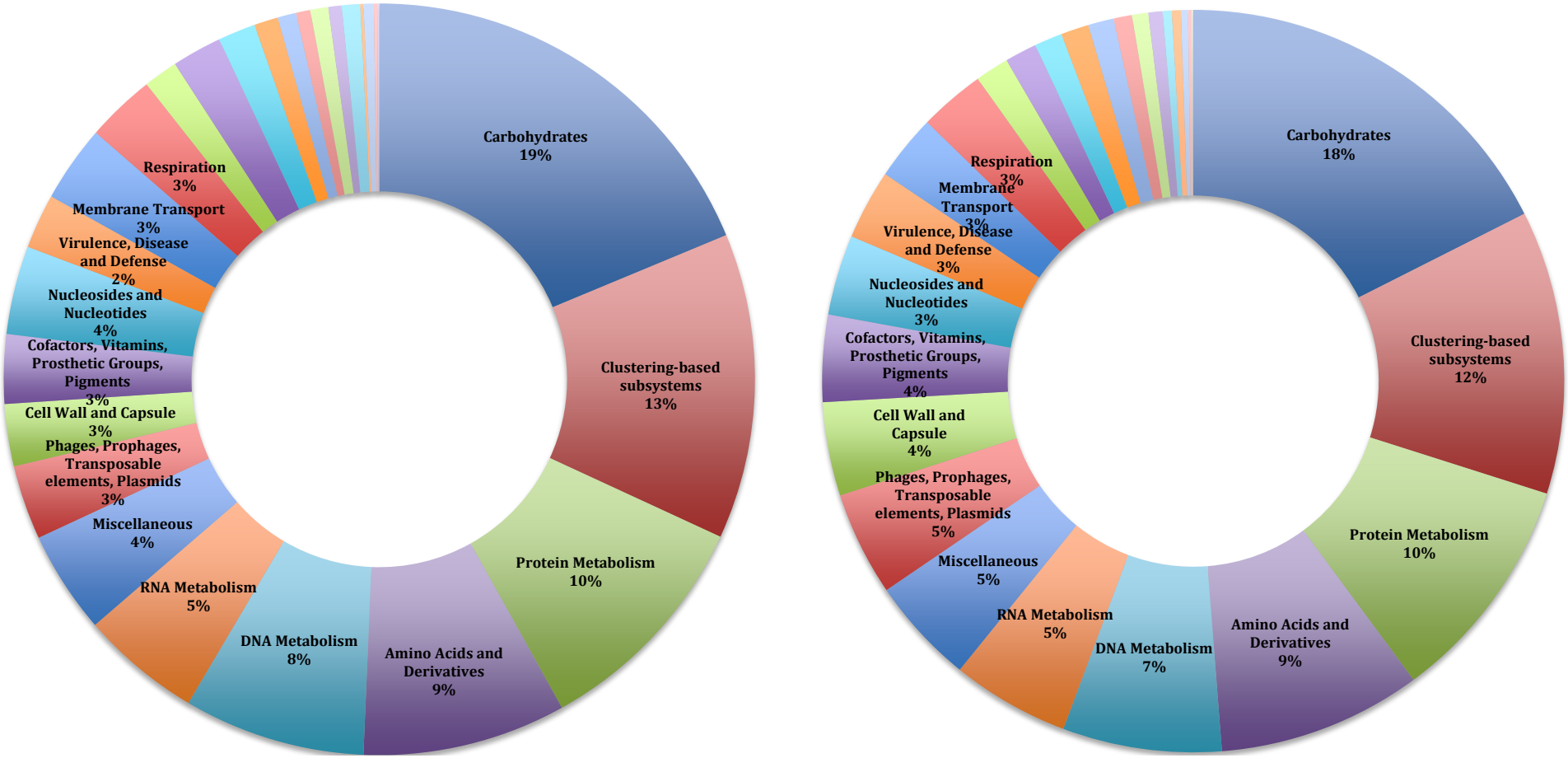


Figure 5-13 Composition of SEED subsystems level 1 functional categories in caeca of the chickens fed sorghum-based diet. Left: Control, Right: H57

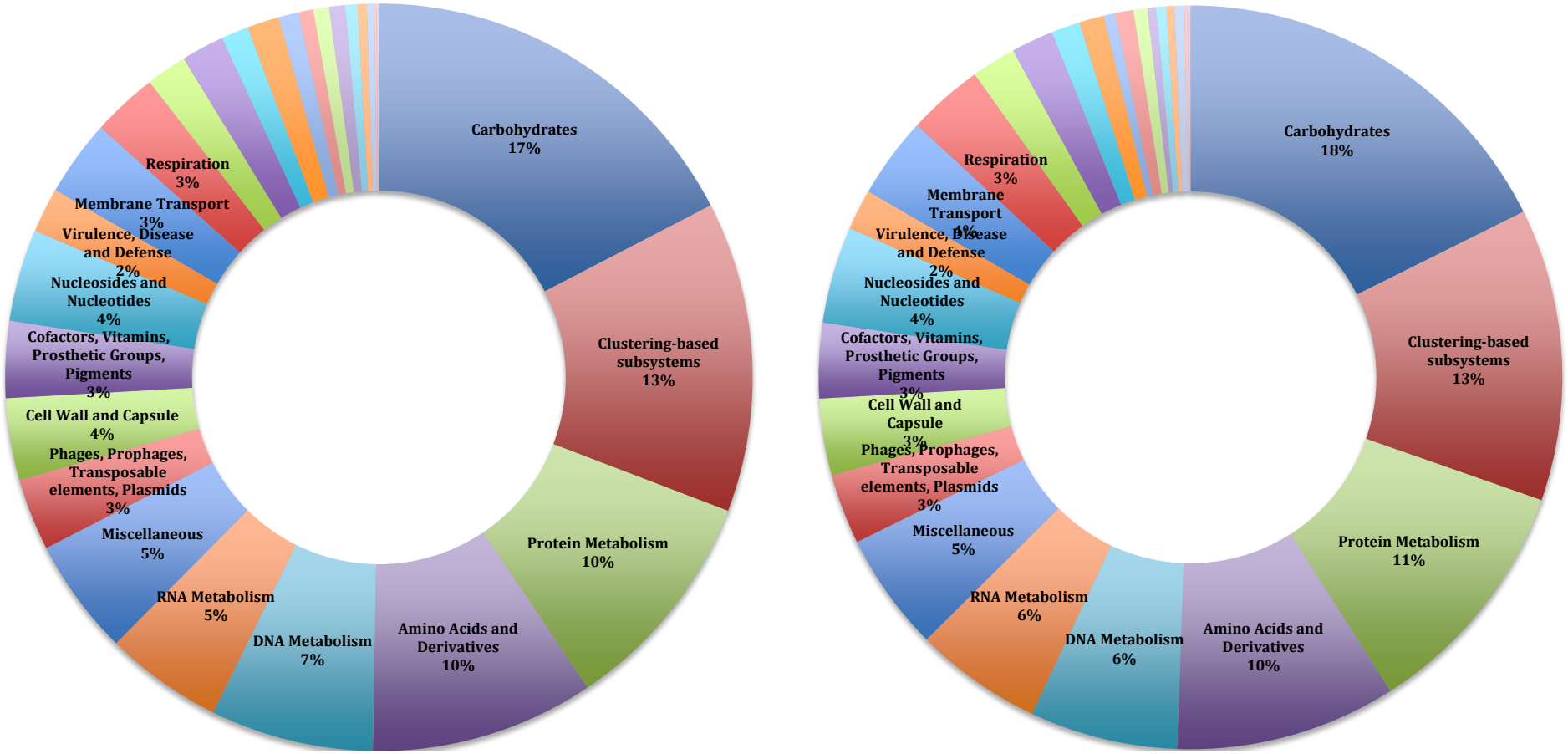


Figure 5-14 Composition of SEED subsystems level 1 functional categories in caeca of the chickens fed wheat-based diet. Left: Control, Right: H57

5.3.2 Metagenome based microbial profiling

5.3.2.1 H57 did not influence microbial community composition

PERMANOVA of the relative abundances of OTUs indicated that there was no significant difference in microbial composition between Control and H57 in either the sorghum or the wheat based diets at day 13 ($P > 0.05$).

5.3.2.2 Microbial community composition differed between diets

However, there was significant effect of feed per se on microbiota as indicated by significantly different ($P = 0.001$) microbial populations between the chickens fed the sorghum-based diet and the wheat based diet (Figure 5-18). As there was no difference between Control and H57 group, indicator species analysis was done for sorghum-based diet and wheat-based diet including both Control and H57. Table 5-10 shows the indicator species which determines the characteristics of the site (Dufrene and Legendre, 1997) with relative abundance >0.1%. A heatmap comparing the relative abundance of OTUs with relative abundance >0.1% is shown in Figure 5-15.

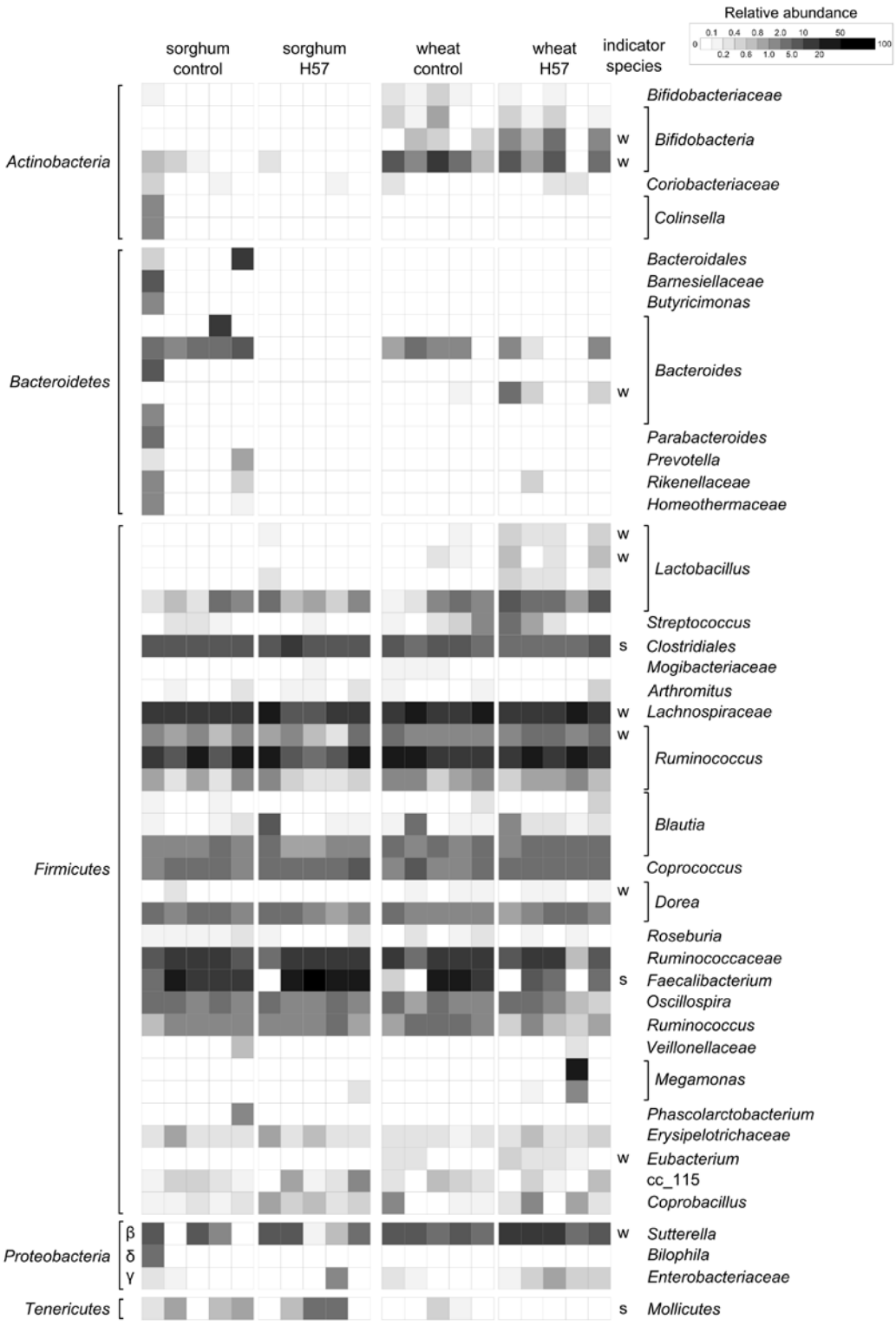


Figure 5-15 Heat map showing OTUs with relative abundance >0.1% in at least one of four treatments (Sorghum Control, Sorghum H57, Wheat Control, Wheat H57). s = indicator species in sorghum-based diet; w = indicator species in wheat-based diet.

Table 5-10 Indicator species (or higher taxonomic classification in case species level classification in not known) in sorghum and wheat based diet with relative abundance >0.1%. f = family, o = order.

Indicator Microbes in the Caeca			
OUT ID	**Sorghum**	**OUT ID**	**Wheat**
193	*Faecalibacterium sp.*	159	*[Ruminococcus] sp.*
188	f_Ruminococcaceae	158	f_Lachnospiraceae
130	o_Clostridiales	229	*Sutterella sp.*
194	Oscillospira sp.	160	*[Ruminococcus] sp.*
171	*Dorea sp.*	169	*Coprococcus sp.*
51	*Bacteroides sp.*	28	*Bifidobacterium sp.*
58	*Bacteroides sp.*	110	*Lactobacillus sp.*
196	*Ruminococcus sp.*	171	*Blautia sp.*
43	o_Bacteroidales	32	*Bifidobacterium sp.*
295	o_RF39	161	*[Ruminococcus] sp.*
165	*Blautia sp.*	35	*Adlercreutzia sp.*
59	*Bacteroides sp.*	124	*Streptococcus sp.*
213	f_Erysipelotrichaceae	220	*Coprobacillus sp.*
218	*cc_115 sp.*	178	*Roseburia sp.*
67	*Parabacteroides sp.*	258	f_Enterobacteriaceae
73	f_Rikenellaceae	34	f_Coriobacteriaceae
75	f_S24-7	56	*Bacteroides sp.*
36	*Collinsella sp.*	116	*Lactobacillus sp.*
69	*Prevotella sp.*	204	*Megamonas sp.*
		216	*[Eubacterium] sp.*
		111	*Lactobacillus sp.*
		117	*Lactobacillus sp.*
		10	*Deinococcus sp.*

Fecalibacterium sp. (OTU ID 193) was the dominant OTU followed by *Ruminococcus* sp. (OTU ID 159) in sorghum based-diet both in Control and H57 fed chickens. Although statistically not significant, the relative abundance of the dominant *Fecalibacterium* OTU increased from 16% in the Control group to 27% in the H57 group. *Ruminococcus sp*. (OTU ID 159) was the dominant OTU in the wheat-based diet fed chickens representing ~21% of the population both in the Control and H57 groups. Figure 5-16 and Figure 5-17 show the composition of microbial populations in the caeca of chickens fed sorghum-based and wheat-based diet respectively. Figure 5-18 compares the microbial composition in the chickens fed sorghum-based diet and wheat-based diet.

A)

B)

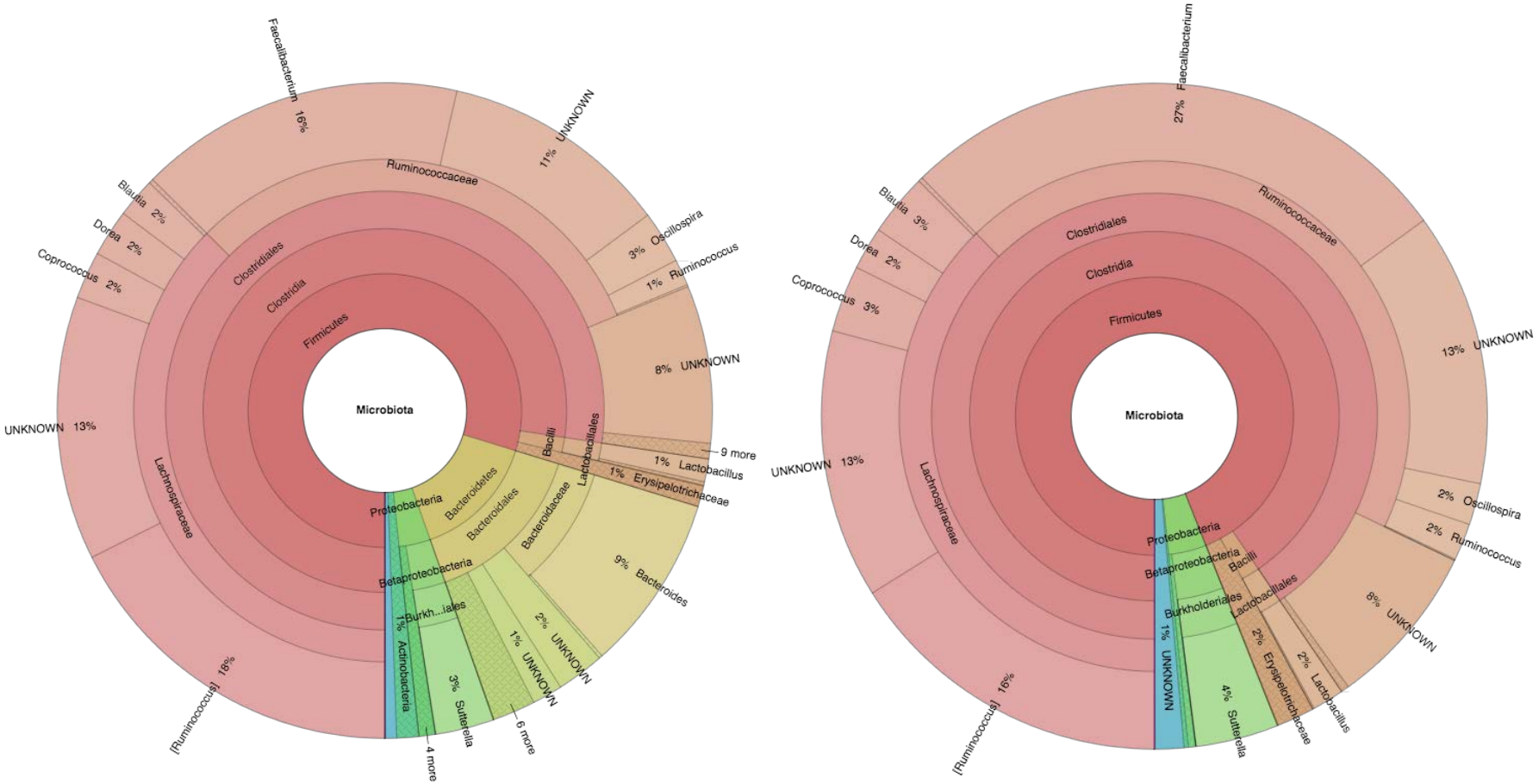


Figure 5-16 Microbiota composition in caecum of the chickens fed sorghum based diet A) Control B) H57

A)

B)

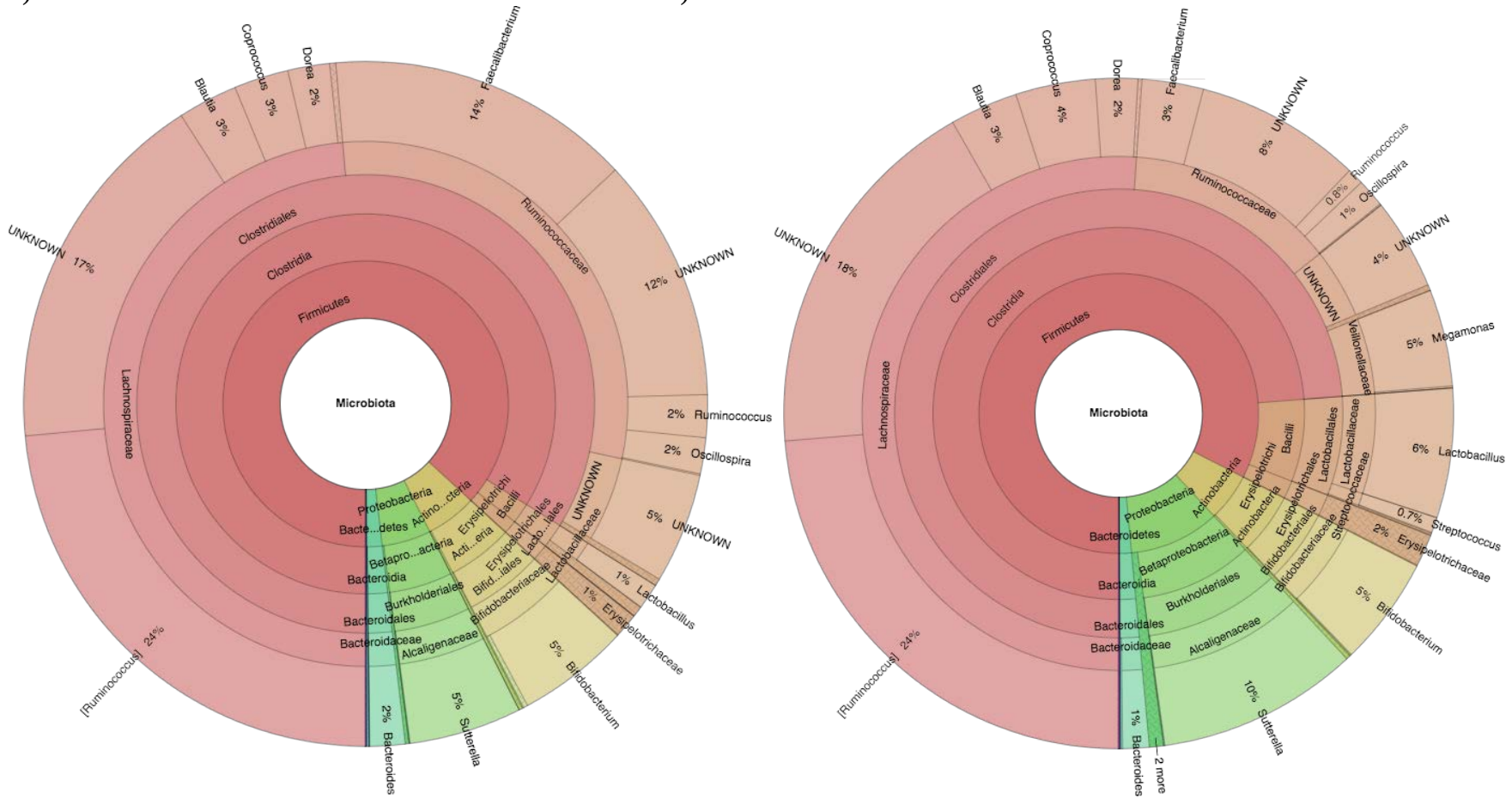


Figure 5-17 Microbiota composition in the caecum of the chickens fed wheat based diet A) Control B) H57

A)

B)

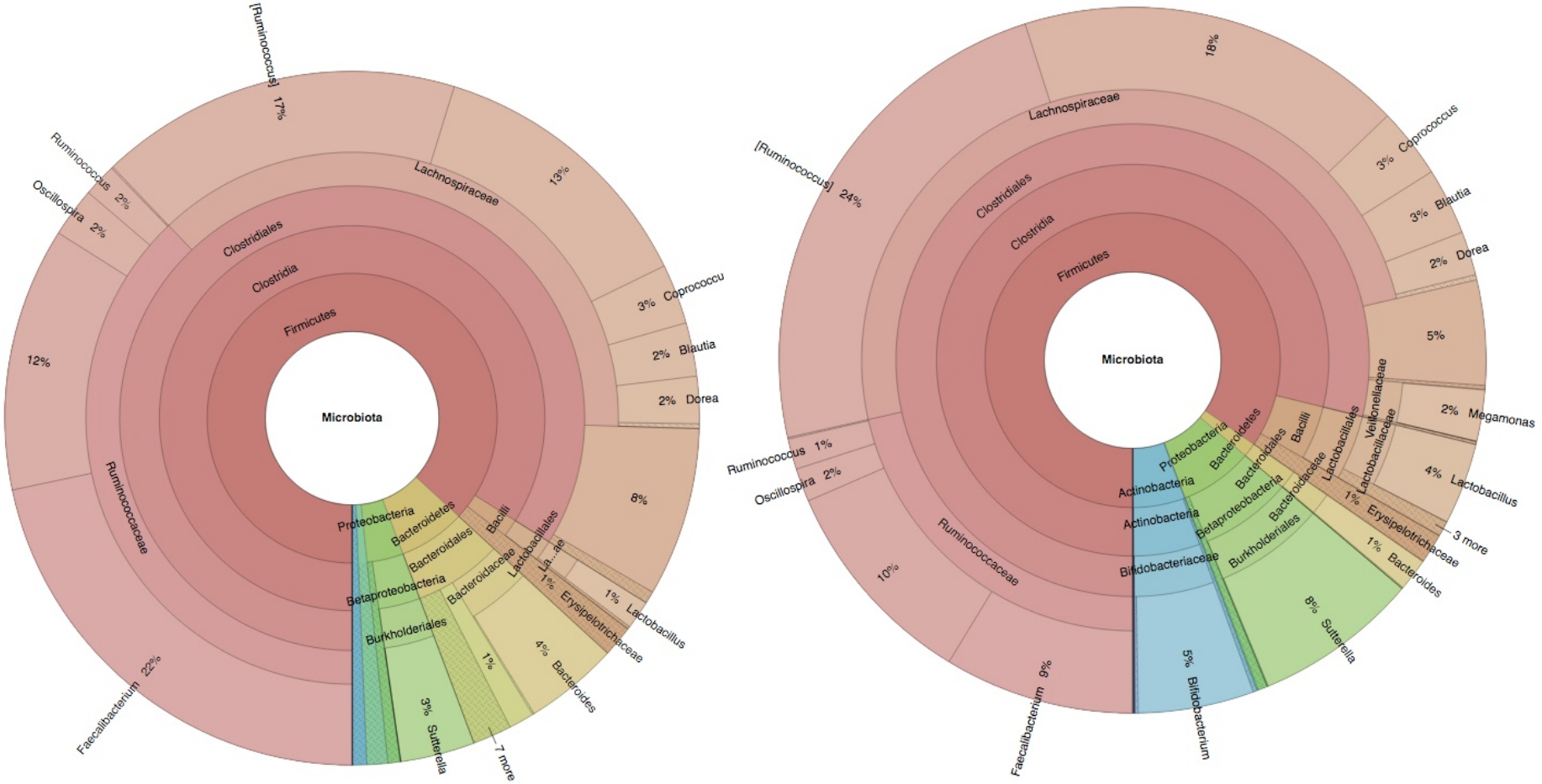


Figure 5-18 Microbiota composition in the caecum of the chickens A) Sorghum-fed birds B) Wheat-fed birds

5.4 Discussion

5.4.1 Microbial functions differs between treatments but dominant functions at higher level of classification are conserved

The inclusion of H57 in the diet appears to influence functional gene profiles in the caeca as indicated by significance differences in the abundance of molecular level functional genes. However, dominant microbial functions at broader level classification remained conserved between treatments. As presented above (section 5.3.1.4), dominant microbial functions (top 3 functional classifications) in the caeca were conserved across diet types (sorghum and wheat) and treatments (Control and H57) up to classification level 3 for annotation based on both KO and SEED subsystems. However, there were several molecular level functions and some other non-dominant higher-level classifications mainly related to fermentation and energy metabolism and microbial virulence factors with significantly different relative abundances between the Control and H57 groups. This indicates that the phenotypic response produced by probiotic H57 could be due to an effect on minor genes affecting less dominant microbial functions. However, it is not known yet how these functions were actually expressed in the caeca as functional potential and their expression could differ (Franzosa et al., 2014). To elucidate this would require a transcriptome and/or metabolome analysis to be undertaken.

The dominant microbial functions in the caeca found in this study (~18% carbohydrates, ~13% clustering based subsystems, ~10% protein metabolism) were similar to previously reported functional group distributions in chicken caeca (Danzeisen et al., 2011). By contrast, carbohydrates (20%), cell wall and capsule (10%), amino acids and derivatives (9%), experimental subsystems (9%) were dominant subsystems level 1 functional categories as reported by (Qu et al., 2008).

Several functional genes were significantly affected due to administration of H57 both with wheat and sorghum based diets. As the abundance of several genes was affected by H57, it is difficult to relate each function to the differences in growth rate and feed efficiency between the Control and H57 groups. Nevertheless, analysis of the differences between H57 and Control treatments at higher level of functional categories could give more insights into the possible mode of action of probiotic H57. We have discussed some important functions affected by H57, which could have direct or indirect impact on body weight gain in poultry.

5.4.2 Impact on bacterial fermentation and energy metabolism

Functional analysis at subsystem level 2 indicated that the percentage of gene sequences responsible for fermentation was significantly higher ($P < 0.05$) in H57 fed birds compared with birds fed the Control wheat-based diet. Wheat contains pentosans as one of the non-starch polysaccharides in significant amounts (Annison, 1991), which could increase the viscosity of digesta thereby slowing passage through the gastrointestinal tract (GIT) (Choct and Annison, 1990, Choct and Annison, 1992b). Non-starch polysaccharides, particularly soluble non-structural polysaccharides present in cereal grains which are non-digestible by the endogenous enzymes of the chicken, can significantly increase the viscosity of digesta, even in small amounts (Annison and Choct, 1991), thereby affecting the nutrient uptake by the birds (Chesson, 2007) because the feed uptake of the birds is slowed by the slow passage through the GIT. This accompanied by reduced nitrogen retention (Choct and Annison, 1992b) and reduced digestibility of amino acids with endogenous amino acid loss (Angkanaporn et al., 1994) may reduce the performance of broiler chickens. Caeca are the most important site for the microbial fermentation of these polysaccharides in the diet of the chicken (Józefiak et al., 2004). Although, the quantitative contribution of caecal fermentation to total energy requirement of the chicken is only about 3–5% (Choct et al., 1992, JøRgensen et al., 1996, Jamroz et al., 2002), fermentative digestion of these polysaccharides may improve the performance of the birds partly by reducing the viscosity and partly by contributing to total energy in the form of SCFAs. Greater abundance of fermentation subsystem genes could lead to a higher rate of fermentation of non-starch polysaccharides with subsequent improvement in the performance of the birds. Schofield (2017) also found that H57 increased the fermentative breakdown of hemicellulose as indicated by increased expression of β-glucosidase, β-galactosidase, α- galactosidase and L-arabinose isomerase in the rumen of sheep fed a palm kernel meal based diet resulting in improved weight gain. Measurement of the concentration of soluble non-starch polysaccharides (NSP) in the diet, intestinal viscosity, and concentration of short chain fatty acids (SCFA) in the intestine would give further insights.

The effect of H57 on microbial energy metabolism of caecal microbes in the chickens fed wheat-based diet was also demonstrated by significantly higher abundance of genes for citric acid cycle (or TCA cycle) and pentose phosphate pathway in the H57 groups. However, there was no difference in the abundance of fermentation subsystem genes in the experiment with sorghum-based diet. This could be due to the differences in the microbial composition between birds fed different diets (wheat vs sorghum).

5.4.3 H57 appears to act through inhibition of bacterial virulence related genes

H57 appears to influence bacterial virulence factors in the gut as indicated by lower abundance of virulence related gene categories in H57 treated birds discussed in subsequent subsections. Microbial virulence factors can stimulate the immune system of the host (Barnes et al., 2002, Hanssen et al., 2004). Activation of immune system requires energy, which could otherwise be utilised for growth and production (Jiang et al., 2010). Measurement of variables that could indicate the stimulation of the immune system for example, level of expression of immunity related genes in the intestinal tissue and/or immunity related specific marker molecules in the blood would be helpful to understand this proposed mode of action of H57. This is discussed in more detail in chapter 7 (section 7.2.1).

5.4.3.1 Regulation of virulence

Abundance of genes responsible for the subsystem "regulation of virulence" was significantly reduced ($P<0.05$) in the birds fed H57 when compared with that subsystem in Control birds in the experiment with sorghum based diet. This could indicate the potential for inhibition of pathogenic microbes by H57 in the intestine or inhibition of virulence factors stimulating immune system of the host.

Several genes, often located in special regions of bacterial DNA called pathogenicity islands are crucial for the bacteria to work as a pathogenic agent (Hacker et al., 1997). Bacteria can modulate the expression of such genes to adapt in particular environments (Thomas and Wigneshweraraj, 2014). Such genes are responsible for the production of virulence factors or pathogenicity determinants, which help the bacteria to successfully adapt in their environment or cause infection or disease with resulting damage to the host (Thomas and Wigneshweraraj, 2014). However, expression of such virulence by the bacteria is a complex process with an array of mechanisms and often depends on several factors (Thomas and Wigneshweraraj, 2014, Mekalanos, 1992). Such virulence factors of the microbes could activate the immunity system of the host (Medzhitov, 2007). Activation of immunity system in vertebrates is an energetically costly phenomenon significantly increasing amount of energy expenditure (Demas et al., 1997, Lochmiller and Deerenberg, 2000, Martin et al., 2003). Immunologically active chickens injected with sheep red blood cells (SRBC) had higher energy expenditure than Control chickens injected with phosphate buffered saline (PBS) as indicated by lower weight gain in spite of higher feed intake in SRBC challenged chickens (Henken and Brandsma, 1982). The energy cost of immune system activation could alternatively be used for growth and production in livestock (Colditz, 2002). Therefore, virulence factors in

intestinal microbes, which can trigger immune system activation, could affect nutrition partitioning with subsequent depression of the growth in the chickens.

5.4.3.2 Capsular and extracellular polysaccharide

Within the sorghum-based diet, genes annotated at functional subsystem level 2, pathways for "capsular and extracellular polysaccharide" were significantly reduced ($P<0.05$) in H57 treated birds compared to birds within the Control diet. Also the proportion of genes encoding the pathway for "gram-negative cell wall components" was significantly lower ($P<0.05$) in H57 group than in the birds with sorghum-based diet without H57. Capsular polysaccharides such as lipopolysaccharides in the cell wall of gram negative bacteria are other important virulence factors of prokaryotic cells (Moxon and Kroll, 1990, Sutherland, 1989). Although these compounds have proven virulence properties such as evasion of phagocytosis and activation of complement system (Moxon and Kroll, 1990), effects of capsular polysaccharides of intestinal microbes on the performance of chickens are yet to be established but stand as an intriguing possible explanation in part for the H57 response.

5.4.3.3 Shiga-toxin

Interestingly, a shiga toxin cluster annotated at subsystem level 2 was found in chickens in the experiment with the sorghum-based diet. The percentage of genes representing shiga toxin cluster were significantly lower ($P<0.05$) in H57 treated birds when compared with birds fed the Control diet. Shiga toxin, which is a cytotoxic protein, is a major virulence factor of shiga-toxin producing *E. coli* (STEC) (Law, 2000). Although ruminants are a major reservoir of these pathogens, chickens are considered less important (Beutin et al., 1993, Chapman et al., 1997, Smith et al., 1991). However, some studies have reported the contamination of poultry meat with STEC O157:H57 (Doyle and Schoeni, 1987, Zhao et al., 2001). *E. coli* O157:H57 is able to colonise the chicken caeca in experimental infection with enterohaemorrhagic *E. coli* (Zhao et al., 1996, Beery et al., 1985).

5.4.3.4 Antibiotic resistance

One of the important virulence factors of the intestinal microbes is the resistance to the antimicrobials. Proportion of genes for the subsystem "resistance to antibiotics and toxic compounds" were significantly lower ($P<0.01$) in H57 group than in the Control group in this experiment.

5.4.4 H57 did not reduce the abundance of genes related to bile salt hydrolase (BSH) activity in the caeca

Probiotics are being considered as an alternative to antibiotic growth promoters in animal diet. Growth promoting effects of antibiotic growth promoters were linked to the reduced effects of bile salt hydrolase in the GIT of chickens (Feighner and Dashkevicz, 1987). There was no effect of H57 on the relative abundance of BSH enzyme (choloylglycine hydrolase) encoding genes for sorghum-based diet in experiment 3. Unexpectedly, relative abundance of choloylglycine hydrolase encoding genes were significantly higher ($P = 0.029$) in birds fed the H57 supplemented wheat based diet than for the birds fed the Control diet. However, most of the bile salts in the intestine are directly recycled through uptake into the enterohepatic circulation from the small intestine (Small et al., 1972). Therefore, BSH activity has more relevance in the small intestine than in the large intestine and caecum.

5.4.5 Effects of H57 on microbial function was different between the chickens fed wheat-based diet and sorghum-based diet

Importantly the effects of H57 on the microbiome are different in wheat based and sorghum based diets. This is probably due to the differences in resident microbes in chickens receiving the two types of diets. As in previous chapters, different groups of microbes were affected by H57 in the three experiments and with different feed types, with different metabolic functions appearing to be affected in the chickens fed different feed types. This reinforces the two major presumptions made in previous chapters that the effects of H57 depends on the structure of resident gastrointestinal microbes and H57 may effect one of several microbes in the intestine i.e. H57 either supresses or stimulates growth of one of several microbes or their metabolic pathways resulting in better performance of birds as an outcome. Also, the hypothesis made in chapter 3 that H57 may overcome the depression of growth in chickens is further supported here from the genomic data in this chapter showing that microbial virulence factors and regulation of virulence were less abundant in H57 treated chickens when compared with that in Control birds fed sorghum-based diet.

Chapter 6 Quantifying Bacillus amyloliquefaciens H57 in the ileum and caecum of chickens

6.1 Introduction

Most *Bacillus amyloliquefaciens* H57 (H57) cells fed to chickens are in spore form. These spores are metabolically inactive and are assumed to act as a probiotic following germination in the gastrointestinal tract (GIT). Nonetheless, evidence demonstrating that ingested H57 spores germinate and multiply in the chicken GIT is limited. In this chapter, we addressed this knowledge gap using real time PCR to quantify H57 in chicken ileum and caecum.

Real-time quantitative PCR (qPCR) is a rapid and robust molecular biotechnological tool, commonly used to quantify the number of copies of a target gene (e.g. a phylogenetic marker or functional gene) in a sample. Quantification of H57 cells in the intestine by real time qPCR may be useful in determining the fate of ingested H57 spores, to assess whether spores have germinated and multiplied in the GIT.

The poly-c-glutamic acid synthase (*pgsB*) gene is responsible for microbial production of polyglutamic acid (PGA) predominantly by *Bacillus* species (Shih and Van, 2001). This gene has been used as a phylogenetic marker to quantify *B. amyloliquefaciens* by qPCR (Yong et al., 2013) using primers and probe specific to *B. amyloliquefaciens*. This chapter outlines the optimisation of the qPCR reaction to detect and quantify *B. amyloliquefaciens* H57 in broiler GIT contents.

6.2 Materials and Methods

6.2.1 Samples and experimental design

Samples for this study were collected from broiler feeding experiments 1 and 2 as described in chapter 3. Briefly, experiment 1 consisted of two treatments (a sorghum based basal diet ± H57) with six replicates per treatment while experiment 2 comprised two treatments (a wheat based basal diet ± H57) with twelve replicates (six in pens and six in cages) per treatment. Ileal and caecal digesta samples were taken from two birds at day 14 and at day 21 from each of the six replicates in experiment 1 and two birds at 35 days of age from six of the 12 randomly selected replicates in experiment 2. Birds were fed starter diet until day 14, then grower diet until Day 28 (until the end of the experiment in experiment 1) and finisher diet till Day 35. The starter and grower diets in experiment 1 were inoculated with $2x10^7$ H57 cells per g of feed while the quantity of H57 in experiment 2 was approximately $6x10^7$ H57 cells per gram of feed in the starter diet and

approximately 10^7 H57 cells per gram of feed in the grower and finisher diets. Thus, the quantities of H57 added in the feed for the samples selected in this study were $2x10^7$ per gram in experiment 1 and 10^7 per gram in experiment 2. Details of digesta sample collection method have been described in chapter 4.

6.2.2 DNA extraction

Genomic DNA was extracted from the digesta samples using the modified repeated bead beating plus column (RBB+C) method (Kawai et al., 2004) using QIAamp Fast DNA Stool Mini Kit as described earlier in Chapter 4. DNA concentration and purity were measured with Multi-Sample Micro-Volume UV-Vis Spectrophotometer NanoDrop 8000 (Thermo Scientific, Wilmington, USA) at an optical density of 260 and 280 nm. The extracted DNA was stored at -20˚C until further analysis.

6.2.3 Preparation of H57 standards

A tenfold serial dilution series of *B. amyloliquefaciens* H57 was prepared from a fresh culture of known numbers of H57. For this, a stock culture of *B. amyloliquefaciens* H57 on nutrient agar (0.5% w/v Peptone, 0.5% w/v NaCl, 0.3% w/v Yeast extract, 1% w/v Glucose, 1.4% w/v Agar) slopes was subcultured on a nutrient agar plate to isolate single bacterial colonies so that descendants of a single cell could be used to prepare the standards. The plate was incubated overnight at 30ºC and a single well isolated colony was picked off with a sterile loop and inoculated into 5 ml of sterile nutrient broth in a Hungate tube and incubated again overnight at 30ºC with shaking in an orbital shaker (Ratek OM11, Ratek Instruments Pty Ltd, Boronia, VIC, Australia) at 160 rpm. Next day, the broth with bacterial growth was poured into 100 ml sterile nutrient broth in a 250 ml Schott bottle and incubated overnight at 30ºC with shaking at 160 rpm.

The concentration of bacterial cells in the resultant culture was determined using a Petroff-Hausser chamber (Hausser Scientific Company**,** Horsham, Australia) following manufacturer's instructions. A 50 µl aliquot of the overnight H57 culture was diluted 1:20 with dilution solution (450 µl of 1x Phosphate Buffer Saline + 500 µl of 100% ethanol) and mixed by vortexing. An aliquot of 100 µl of the diluted cell suspension was placed on a clean and dry Petroff-Hausser chamber's covering the counting grid and covered by a coverslip. The chamber was placed on a light microscope (Olympus BH-2, Olympus Corporation, Japan) stage cells allowed the cells to settle for 10 min following which the bacterial cells were counted. The average of three counts was used to calculate the number of cells per ml.

Two 10-fold serial dilutions (one for ileal and the other for caecal contents) of H57 in TE buffer containing H57 cells from $5x10^9$ to $5x10^3$ cells per ml were prepared. To account for the presence of any PCR inhibitors in the digesta, 0.2 g of ileal or caecal content (for their respective standard series) from control birds was added to each tube of the dilution series. This ensured the similar reaction environment for standards and unknown samples making them comparable (Ouwerkerk et al., 2002). The dilution series in the Eppedorf tubes were stored at -80ºC prior to DNA extraction. DNA was extracted from 1 ml aliquots of each tube using modified repeated bead beating plus column (RBB+C) method (Kawai et al., 2004) using QIAamp Fast DNA Stool Mini Kit and eluted in 200 µl final volume as described in chapter 4.

6.2.4 qPCR Primers and probes

Many *Bacillus* species including *B. amyloliquefaciens* produce poly-γ-glutamate (Luo et al., 2016, Shih and Van, 2001). Recently, a primer pair and probe specifically targeting the poly-γ-glutamic acid synthesis gene (pgsB) of *B. amyloliquefaciens* has been designed (Yong et al., 2013). Details of the primers and probe used in this thesis are listed in Table 6-1.

Table 6-1: Primers and probe targeting the pgsB gene of *B. amyloliquefaciens* (Yong et al., 2013).

Primers	Sequence (5' -> 3')	Molecular wt.	GC%	Melting temperature (°C)
pgsB726-f	TGGCGCCATGAGAATCCT	5500	55.55	66.9
pgsB791-r	GCAAAGCCGTTTACGAAATGA	6463	42.86	65.8
pgsB-probe	[1]FAM-CCGCTGCTCAGCACGAAGGAGC-TAMRA[2]	78910 µm	68.18	76.3

[1] FAM (6-carboxy-fluorescein), [2]TAMRA (6-carboxy-tetramethylrhodamine)

6.2.5 Optimising the annealing temperature by thermal gradient PCR

The temperature at which oligonucleotide primers anneal to a DNA template (i.e. annealing temperature) is an important parameter affecting the specificity of a PCR reaction. The original study which developed the primers and probe set was based on DNA samples extracted from pure

broth culture and solid-state fermentation product of *B. amyloliquefaciens* (Yong et al., 2013). In contrast, our samples contained DNA from a multitude of gastrointestinal microbes together with possible PCR inhibitors present in the broiler gut. In addition, PCR master mix and PCR machine used in our study was different from those used by Yong et. al (2013). Therefore, we tested modifications from the published method with a thermal gradient PCR to obtain optimal PCR amplification conditions.

PCR reaction mix (25 µl) was prepared in triplicate by mixing 10 µl of 1xRealMasterMix Probe (5 PRIME, Gaithersburg, MD, USA), 1.25 µl of 5 µM forward and reverse primers, 5 µl of template DNA sample and ultrapure water to make the final volume 25 µl. Thermal gradient PCR was carried out in Bio-rad C1000 thermal cycler (Bio-Rad, Singapore) under the following reaction parameters.

- 95ºC for 30 s (for initial denaturation and activation of FastStart Taq DNA polymerase)
- 34 cycles of
    - 95ºC for 5 s
    - Gradient 55/70ºC (70ºC, 69.2ºC, 67.5ºC, 64.5ºC, 60.9ºC, 58.0ºC, 56.0ºC, 55.0ºC) for 30 s
- Hold at 4ºC

Amplified DNA was visualised by agarose gel electrophoresis. One per cent (w/v) agarose solution was prepared in 1X TBE (Tris-Borate-EDTA) buffer with 10µl/100ml of fluorescent nucleic acid gel stain (GelRed^{TM} 10000x, Biotium, Hayward, CA, USA). The solution was smoothly poured into an electrophoresis tank with combs fitted in a tray and left for 30 minutes to set. The gel was placed in an electrophoresis tank with 1X TBE buffer and the combs were carefully removed to make wells to load the samples. The first well of each row was loaded with 5 µl of 1 kb ladder (Bioline Aust. Pty. Ltd., Alexandria NSW, Australia) diluted into 1:4 with 5X loading buffer. The PCR products were then loaded into the wells by mixing 5µl of the products with 3 µl of 5X loading buffer. The electrophoresis was run at 95 volts and 400 mA for 45 minutes and visualised by using Gel Doc™ XR+ System (Bio-Rad Laboratories, Philadelphia, USA) on completion.

6.2.6 Optimisation of primers and probe concentration

Combinations of six concentrations of primers (100 nM, 150 nM, 200 nM, 250nM, 300 nM, and 900 nM) and four concentrations of probe (50 nM, 100 nM, 150 nM, 200 nM) were tested by preparing 24 different reaction mixes. These 24 different reactions were run in triplicate in Rotor-

Gene 6000 (Corbett Research, Australia) and analysed with Roter_Gene Q software version 2.3.1.49 to find the lowest threshold cycle, the cycle number at which the fluorescence in the reaction can be detected from the background. The concentration of primers and probe that results lowest threshold cycle, would be the optimum concentration of primers and probe.

6.2.6.1 qPCR of the ileum and caecum digesta samples gDNA

The qPCR reaction was carried out in 25 µl volumes in 0.1 ml thin walled strip tubes (QIAGEN, Velno, The Netherlands). Each sample was amplified in duplicate in a Rotor-Gene 6000 (Corbett Research, Australia) with two non-template control reactions in each run. The PCR reaction consisted 10 µl of 1X RealMasterMix Probe (5 PRIME, Gaithersburg, MD, USA), primers and probe (optimised concentration as in section 6.2.6), 5 µl of sample gDNA diluted 1:10 in ultrapure nuclease free water and ultrapure water to make total volume 25 µl. The amplification parameters were 95°C for 30 s (denaturation), 40 cycles of 95°C for 5 s (denaturation) and 60.9°C (optimised as in section 6.2.5 above) for 34 s (annealing and extension). Respective H57 standard series from 10^9 cells/ml to 10^4 cells/ml prepared as in section 6.2.3 were amplified with the samples in each run. The quantitation report was prepared by analysing the threshold cycle value (CT) with Rotor-Gene Q software version 2.3.1.49.

6.3 Results

6.3.1 Optimization of annealing temperature

The annealing temperatures 56ºC , 58ºC and 60.9ºC resulted in good yields of the PCR product of correct size as indicated by clear bands in agarose gel electrophoresis (Figure 6-1). As higher annealing temperatures have greater specificity (Wu et al., 1991) and typically annealing temperature is about 3 to 5ºC below the melting temperature of primers (i.e. the temperature at which the bonds between the oligonucleotide primer and the template are denatured), 60.9ºC was chosen for further qPCR reactions based on gradient PCR result and calculated melting temperature of the primers.

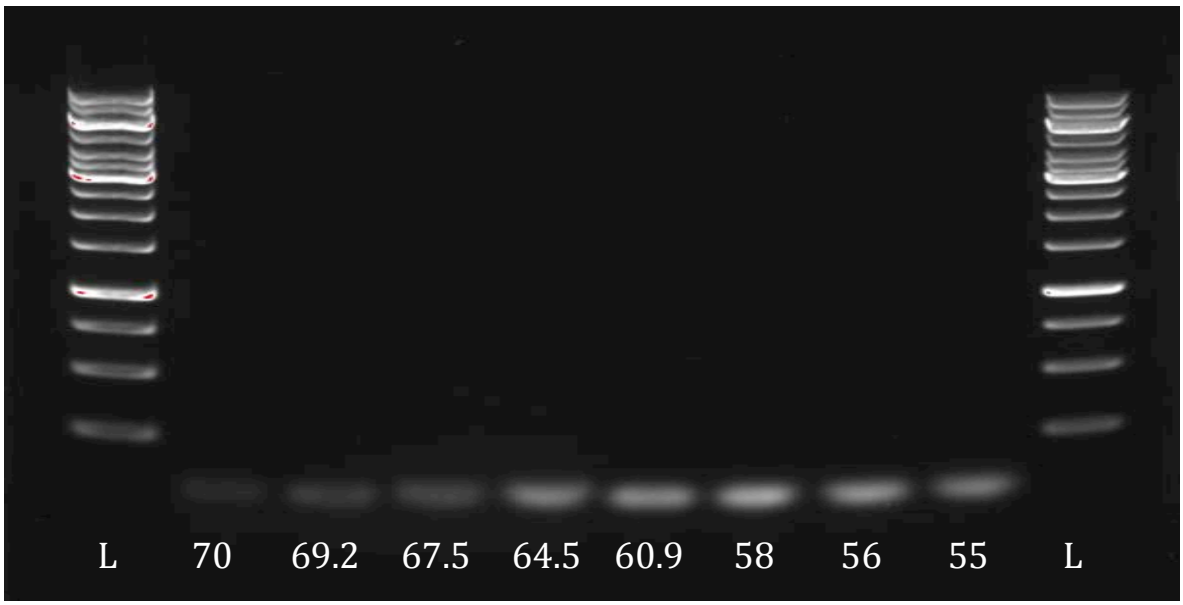


Figure 6-1 Optimization of annealing temperature by thermal gradient PCR. L = ladder

6.3.2 Optimization of primers and probe concentration

Among 24 different combinations of primer and probe concentrations tested, 250 nM of primers and 150 nM of probe resulted in the lowest threshold cycle (Ct) (data not shown). Therefore, 250 nM of primers and 150 nM probe was used as the optimum combination for qPCR reactions.

6.3.3 Optimization of background signal and Ct values

Standards prepared with ileum or caecal digesta were used to offset the effect of any inhibitory factors present in GIT. In addition, "Dynamic Tube" normalisation, which is regarded as the most precise method for the normalisation of qPCR data, was used to optimise the background signal and the Ct values (Mallona et al., 2011). This method uses the second derivative of the sample to assign the baseline for each sample and the background fluorescence is averaged from the first cycle to this baseline for each reaction separately (Mallona et al., 2011).

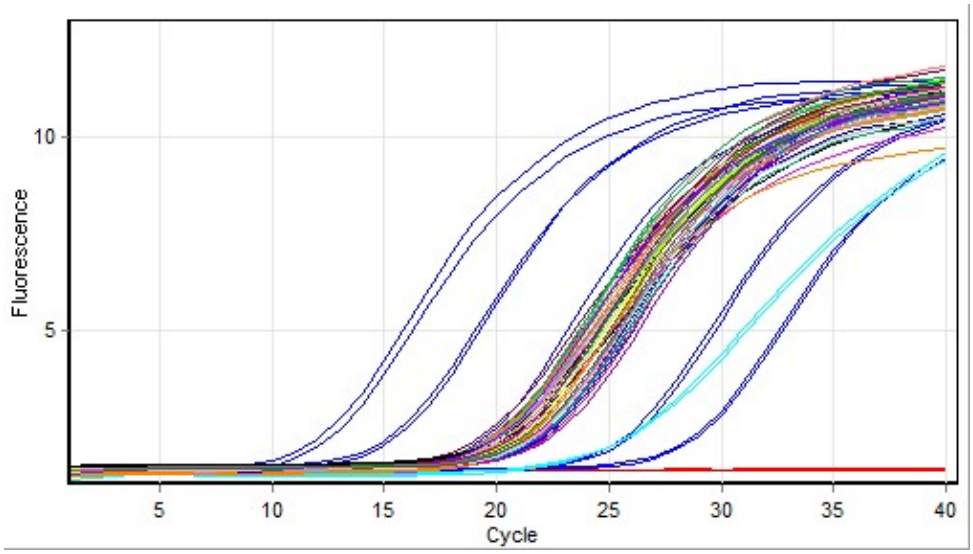
6.3.4 Standard curves and reaction efficiency

Standard curves in each qPCR run were generated by using H57 standards consisting of tenfold serial dilutions of DNA representing H57 cells from 5×10^9 cells/ml down to 5×10^3 cells/ml. The linearity of the relationship between concentration of H57 and Ct values was tested by plotting the values. There was good linearity between concentration and Ct from 5×10^9 cells/ml to 5×10^4. Therefore, this range of H57 cell concentrations were subsequently used.

6.3.5 qPCR with samples from ileum of H57(+) birds

The H57 tenfold serial dilution series for ileum gave a good linear correlation (R^2 = 0.998) and a high amplification efficiency (E = 100%) (Figure 6-2).

a)



b)

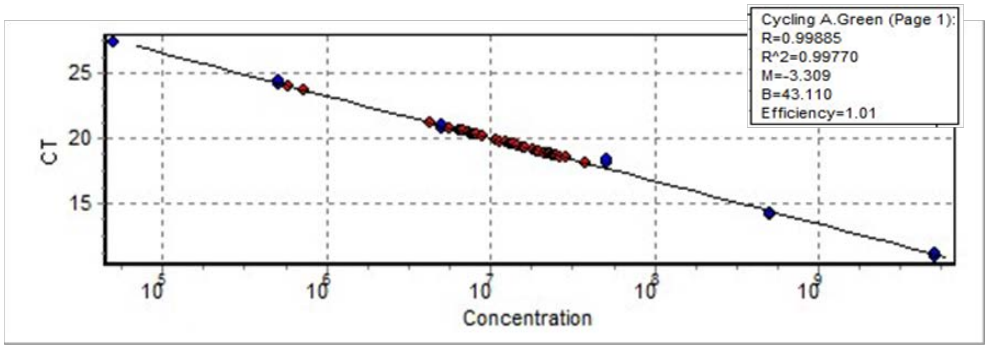
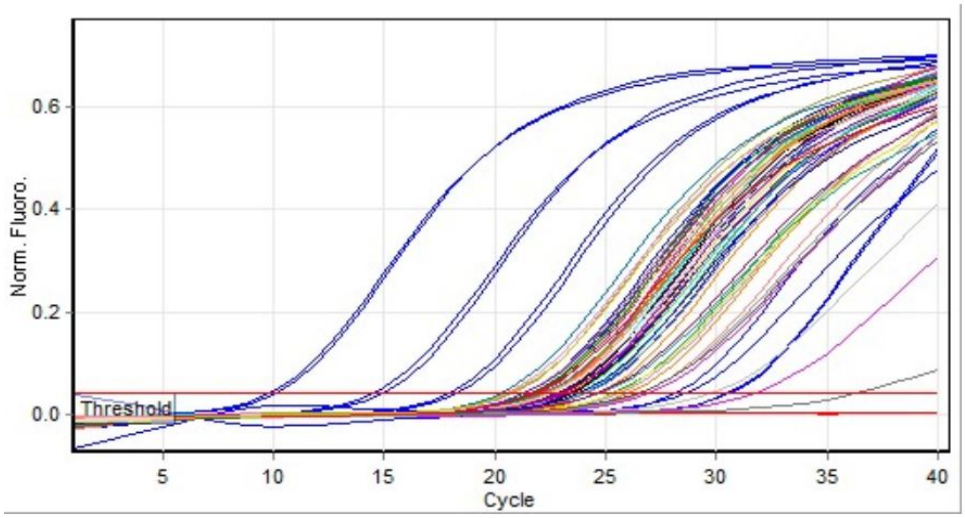


Figure 6-2: a) Amplification curves for samples from ileum of H57+ birds. Blue = standard series. Red = Non-template control. Other colours = samples. b) Standard curve (regression line) generated with *B. amyloliquefaciens* H57 standards series (5×10^9 – 5×10^4) with ileum content. CT = Threshold Cycle. R and R^2: Correlation coefficients. M: Slope of the standard curve. B: Intercept with the ordinate. Efficiency: Reaction efficiency.

6.3.6 qPCR of samples from caeca of H57(+) birds

Results from caecal samples gave good linearity (R^2 = 0.99) and amplification efficiency (97%) (Figure 6-3).

a)



b)

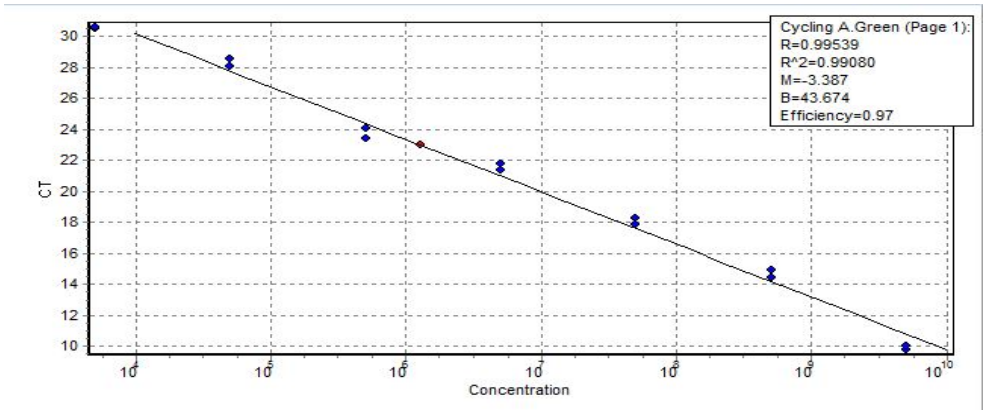


Figure 6-3: a) Amplification curves for qPCR run with samples from caeca of H57+ birds. Blue = standard series. Red = Non-template control. Other colours = samples b) Standard curve (regression line) generated with *B. amyloliquefaciens* H57 standards series (5×10^9 – 5×10^4) with caecal content. CT = Threshold Cycle. R and R^2: Correlation coefficients. M: Slope of the standard curve. B: Intercept with the ordinate. Efficiency: Reaction efficiency.

6.3.7 qPCR run with samples from ileum and caecum of control birds

As the samples from both ileum and caecum of the birds fed the diet without H57 were amplified in a single qPCR run, H57 standard series from 5×10^9 to 5×10^3 cells/ml prepared in TE (Tris-EDTA) buffer was used for qPCR. The standard curve was well correlated (R^2 = 0.962) with a high amplification efficiency (E = 97%) (Figure 6-4).

B. amyloliquefaciens is generally not present in the GIT of the chickens. Therefore, amplification of target sequence was not expected in control samples. Although there were some non-zero values for the quantity of *B. amyloliquefaciens* H57 in both the ileum and caecum samples of control birds in

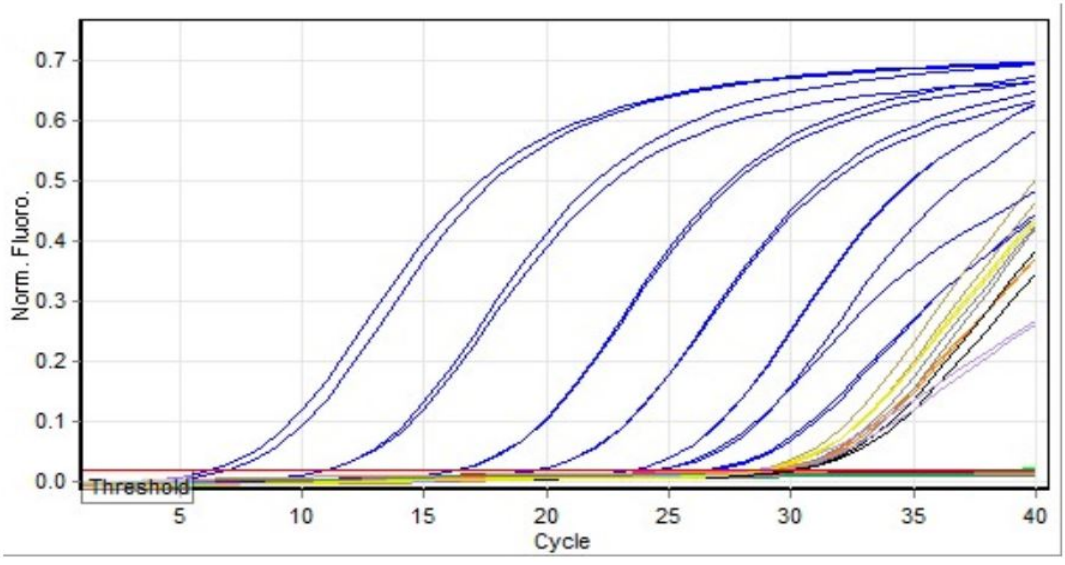
both experiments, these values were below the detection limit of the assay (data not shown) i.e. below the concentration in which CT values and concentration had linear relationship (<5×10^3 cells/ml). Some late cycle amplification in a few of the control birds (Figure 6-4a) were assumed to be due to non-specific amplification of the target sequence.

6.3.8 Enumeration of H57 in the GIT of chicken

The average number of *B. amyloliquefaciens* H57 cells in the ileum of H57 (+) birds in experiment 1 - week 2 (day 14) was 1.1×10^7 cells/g while in week 3 (day 21) it was 1.05×10^7 cells/g of digesta (Table 6-2). There was no significant difference ($P > 0.05$) in the numbers of H57 in the ileum between week 2 and week 3. The average quantity of H57 in the ileum at day 35 in experiment 2 is 3.75×10^6 cells per gram of digesta (Table 6-3).

The average number of *B. amyloliquefaciens* H57 in the caecum of H57(+) birds in experiment 1 - week 2 (day 14) and week 3 (day 21) were 2.17×10^6 cells/g and 1.4×10^6 cells/g of digesta respectively (Table 6-2). The difference in the numbers of H57 between week 2 and week 3 was not significant ($P > 0.05$). However, the differences in the population of H57 between ileum and caecum were statistically significant ($P < 0.05$). The average quantity of H57 in the caeca at day 35 in experiment 2 was 1.27×10^5 cells per gram of digesta (Table 6-3).

a)



b)

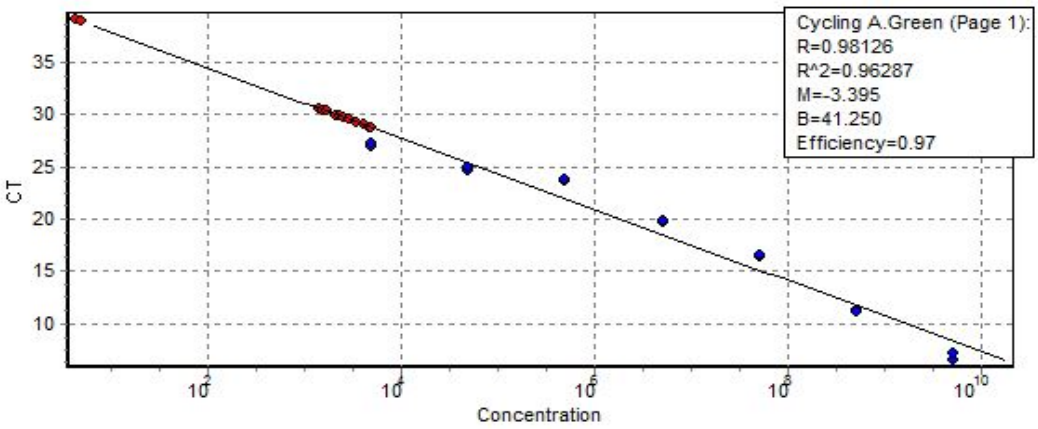


Figure 6-4: a) Amplification curves for qPCR run with samples from ileum and caecum of control birds. Blue = standard series. Red = Non-template control. Other colours = samples b) Standard curve (regression line) generated with *B. amyloliquefaciens* H57 standards series (5×10^9 – 5×10^3) in TE buffer. CT = Threshold Cycle. R and R^2: Correlation coefficients. M: Slope of the standard curve. B: Intercept with the ordinate. Efficiency: Reaction efficiency.

Table 6-2: Population density of *B. amyloliquefaciens* H57 in the ileum and caecum of H57+ chickens on day 14 and day 21 in experiment 1. Numbers are the average of two birds per replicate.

Replicates	Day	Ileum (per gram of digesta)	Caecum (per gram of digesta)	Quantity added in feed (per gram)
1	14	1.21×10^7	2.25×10^6	2×10^7
	21	1.07×10^7	4.57×10^6	2×10^7
2	14	1.05×10^7	7.61×10^5	2×10^7
	21	6.64×10^6	1.82×10^6	2×10^7
3	14	1.17×10^7	9.92×10^5	2×10^7
	21	1.09×10^7	1.94×10^6	2×10^7
4	14	6.75×10^6	1.03×10^6	2×10^7
	21	1.16×10^7	1.17×10^6	2×10^7
5	14	1.18×10^7	7.09×10^5	2×10^7
	21	1.55×10^7	8.42×10^5	2×10^7
6	14	1.04×10^7	5.03×10^5	2×10^7
	21	1.03×10^7	2.70×10^6	2×10^7

Table 6-3: Population density of *B. amyloliquefaciens* H57in the ileum and caecum of H57+ chickens at day 35 in experiment 2. Numbers are average of two birds per replicate.

Replicates	Day	Ileum (per gram of digesta)	Caecum (per gram of digesta)	Quantity added in feed (per gram)
1	35	3.74×10^6	2.31×10^5	10^7
2	35	2.75×10^6	1.22×10^5	10^7
3	35	4.29×10^6	1.58×10^5	10^7
4	35	3.72×10^6	2.66×10^4	10^7
5	35	4.58×10^6	1.38×10^5	10^7
6	35	3.42×10^6	8.62×10^4	10^7

6.4 Discussion

The numbers of H57 detected, as indicated by copy number of pgsB gene in digesta, were consistently lower than the number added into the feed. Therefore, we can presume that H57 has not multiplied noticeably in the intestine, and any cell division is about equivalent to or less than cell death. Also, lower numbers of H57 in the digesta tends to indicate the death of a small proportion of cells in the intestine, although higher moisture level in digesta than in feed may have been a factor.

Significant effects of feeding H57 on production performance and intestinal microbiota populations and function might be suggestive of spores germinating in the intestine, as the spores would need to germinate to be metabolically active. Sequencing of the H57 genome indicated that this bacterium has the genetic potential to grow in anaerobic conditions (Schofield et al., 2016). Due to the relatively short (a few hours) transit and retention time of digesta in the chicken GIT (Shires et al., 1987), probiotic spores would need to germinate soon after ingestion to produce a marked metabolic impact (Jadamus et al., 2001). Even though *Bacillus* can survive for long periods as a spore, these can be triggered to germinate within a short period of time (within a few minutes to 24 hours) by different factors among which nutrients such as amino acids, sugars or nucleosides have been implicated (Moir, 2006, Setlow, 2003). Chicken diets are generally dense in nutrients, which could trigger germination of bacterial spores present in feed.

Previous studies have demonstrated that *Bacillus* spore can germinate in the GIT of chicken. Cartman *et al.* (2008) used *Bacillus subtilis* vegetative cell specific reverse transcriptase (RT)-PCR targeting a unique *rrnO-lacZ* fusion gene and demonstrated that *B. subtilis* spores germinated in the intestine of chickens. In this study, the number of vegetative cells was greater than spores in all sections of the GIT after 20 hours following oral administration of *Bacillus subtilis* spores to

chickens. However, *Bacillus* spores were unable to colonize the gastrointestinal tract of chickens as the number of *Bacillus* cells in different sections of the GIT decreased with time after a single large oral dose (10^9 spores) was administered to day old, specific pathogen-free chickens (Cartman et al., 2008). In another study, Jadamus *et al.* (2001) showed that about 90% of the ingested spores of *Bacillus cereus var. toyoi* in feed germinated within 30 minutes in the crop while 99% of spores germinated by the time they reached the proventriculus.

Probiotic bacteria need to survive the acidic environment of the gastric stomach to reach the lower GIT. Germination of most of the orally administered spores before they reached the stomach, and detection of the vegetative cells in the lower GIT, as discussed above indicates a tolerance of *Bacillus* vegetative cells to acid conditions in the proventriculus.

However, some of bacterial cells may lose viability during germination or during transit from the small intestine to the caeca. In our experiments the population of H57 in the caeca is less than the population in the ileum based on number of cells per gram wet digesta. Jadamus *et al.* (2001) also found a similar result, that the total CFU of orally administered *Bacillus cereus var. toyoi* was less in caeca than in the ileum. Only finely ground, low molecular weight and non-viscous particles in the small intestine enter the caeca (Svihus et al., 2013). Therefore, some H57 cells could be directly shed to the faeces without going to the caeca resulting in lower numbers of H57 cells in the caeca.

Some *Bacillus* probiotics cannot only germinate, but can also "re-sporulate" when the vegetative form is fed to the birds. Oral administration of vegetative cells of *Bacillus cereus var. toyoi* led to the *in vivo* sporulation of *Bacillus* cells in the chicken intestine resulting in the detection of the spore form in all sections of the GIT two hours after ingestion (Jadamus et al., 2001). Harsh environmental conditions and/or the anaerobic conditions of the GIT might have triggered vegetative cells to sporulate. An immunological study by Tam *et al.* (2006) demonstrated that *B. subitilis* spores can germinate and "re-sporulate" in the intestine of mice.

These findings indicate that H57 spores may germinate in the intestine, but undergo minimal cell division and multiplication. The newly germinated cells may also re-sporulate due to the harsh environmental conditions that prevail in the intestine. A proportion of cells might have lost their viability in this process leading to the decreased quantity in the caeca as compared to ileum.

Chapter 7 General discussion and implications of the research

Administration of a novel *Bacillus amyloliquefaciens* strain H57 (H57) through feed improved the growth rate and feed use efficiency in broiler chickens, but the results varied between experiments and diets. This study indicated that H57 did not noticeably multiply in the intestine of the chickens but modulated the intestinal microbial profile and their function. These aspects of the research are further discussed below along with the possible mode of action(s) of H57 and potential future research directions.

7.1 General considerations

7.1.1 Variability of results

While possible modes of action of the probiotic H57 discussed below provide a direction for further studies, the most confounding aspect of the results of this study is the inconsistency of results between experiments and the interaction of H57 with diets. There was a significant effect of H57 both on intestinal microbiota (both in ileum and caeca) and growth rate in experiment 1. In Experiment 2, the microbiota was significantly affected by H57 (but less prominently than in experiment 1) with a shift in relative abundance of some OTUs in the caeca but there was no effect on growth rate. Conversely, growth rate was significantly increased by H57 in experiment 3 with a less prominent effect on the microbial profile than in experiment 1 (details in **chapter 4**).

While there was a significant correlation (positive or negative) between body weight and relative abundance of some OTUs affected by H57 in experiment 3, other OTUs affected by H57 had no correlation with body weight. Therefore, some of the OTUs might have directly contributed to the differences in the body weight while others may not have had a direct influence on body weight gain even though they were significantly affected by H57. Even though there were no differences in microbial profiles in the caeca in experiment 3 at day 13, microbial functional capacity as indicated by differential abundance of functional genes was significantly different between Control and H57 birds. However, by day 13 there was a significant increase in body weight with H57. Therefore, it appears that in some situations the response to H57 was mediated not by the microbial population *per se* but their functional potential to achieve a phenotypic response. Changes in the metagenomic profile but not in microbial community profile in experiment 3 indicated that the differences in genetic potential can be attributed to variation in microbial community differences below genus or species level, for example, different strains of same species having different levels of virulence due

to the presence or absence of pathogenicity islands (Salama et al., 2000, Medini et al., 2005). These observations indicated that the effect of H57 on growth are not necessarily associated with an effect on the microbial profile but rather on which microbes or microbial functions in particular were affected. These particular microbes or functions, which when altered can have a positive effect on the growth rate of the host, could encompass a range of microbes or functions which may differ between different experiments and diets. Two different batches of chicks with similar diets (sorghum based diets) developed different microbial populations at day 21 (**chapter 4**). This may be due to differences in the "seed microbes" initially colonizing the gut, which resulted in different resident microbes by day 21. Therefore, the difference in response to H57, positive in Experiment 1 and none in Experiment 2 may be due to variability in the resident microbes in the gut resulting from the different batch of chicks and the different source/batches of feed ingredients between experiments.

In experiment 3, birds fed sorghum and wheat mixed diet had higher growth rate than the birds fed the feed based on only sorghum or wheat. In contrast to our result, a recent study showed that birds with a sorghum based diet grew better than birds fed sorghum and wheat mixed diet (Crisol-Martínez et al., 2017). In this study, the difference in birds' performance between diets was associated with the microbial profile in the caeca. This result further reinforces our hypothesis that a bird's performance and/or the effects of probiotics and feed depends on the resident microbes in the gut prior to the introduction of probiotic bacteria.

However a recent study has reported that microbial population structure in the caeca of chickens is highly variable, varying from batch to batch even for the same breed of chicks, from the same hatchery and raised on the same diet (Stanley et al., 2013). The authors have suggested that this variation is due to "the lack of colonisation of the chicks by maternally derived bacteria". The modern poultry industry hatches chicks in an environment with minimal contamination by microbes. Therefore, the development of resident microbial populations in the gut may depend on the microbes they are exposed to during the initial days of life, from the room and microbes in the feed. This could be a major reason for variability of results in the experiments reported in this thesis. Effects of probiotics in animal agriculture are highly variable (**chapter 2**) (FAO, 2016). These inconsistencies in results may be partly due to variation in the innate intestinal microbial profile. Furthermore, the GIT of the chicken also consists of protozoa, fungi, bacteriophages and other viruses together with bacteria (Yeoman et al., 2012, Saengkerdsub et al., 2007a, Saengkerdsub et al., 2007b, Apajalahti et al., 2004). Although this study focussed in bacterial population, which is believed to be the primary microbial population in the GIT, it could be further insightful to study the effects of H57 on microbes other than bacteria in future studies.

7.1.2 Germination and growth of *B. amyloliquefaciens* H57 spores in the avian GIT

The extent of spore germination and multiplication of *B. amyloliquefaciens* in the presumed anaerobic gastrointestinal tract (GIT) of chickens remains unresolved. Although *B. amyloliquefaciens* is regarded as an aerobe, genome sequencing showed that H57 has the capacity to grow in a low PO_2 using nitrate as an electron acceptor (Schofield et al., 2016). The number of H57 cells present in the digesta sample of birds was determined by a real time quantitative PCR (qPCR) method (**chapter 6)**. This method unfortunately cannot distinguish between spore and vegetative or dead (recent) cell DNA. However the qPCR analysis showed that there was no major multiplication of vegetative cells in the GIT and if spores had germinated then the vegetative growth was just sufficient to maintain the inoculum population size. In the H57 inoculum, cells constitute less than 20% of the material spun down from the production fermentation from calculations based on the wet weight of material spun down from the fermenter, freeze dry weight of inoculum, cell number and dry weight (**chapter 3**). Thus the response to H57 could be mediated by the bacterium *per se* in some form or from products of growth in culture, such as extracellular materials, e.g. enzymes such as amylase and protease; lipopeptides such as surfactin, fengycin, iturin; polyketides; and signalling molecules affecting hormone production (Schofield et al., 2016), resulting in the modification of GIT microbiome and enabling a better utilisation of the nutrients present in feed.

7.2 Possible modes of action

7.2.1 Microbial virulence factor and possible role of immune system activation for growth depression

It appeared from this study that H57 helped the underperforming chickens to overcome slower growth rate. One of the notable insights from three feeding experiments as discussed in **chapter 3** is that H57 has an effect only when the growth rate was below the performance objectives, and this was interpreted as a growth depression in the chickens, which H57 ameliorated.

Microbial virulence factors can trigger the immune system of the host to mount a response mediated through pattern-recognition receptors (Medzhitov, 2007). For example, lipopolysaccharides (LPS) (virulence factors in the cell walls of gram negative bacteria) can trigger an acute phase immune response in the host. This results in the activation of toll-like receptors and stimulation of macrophages to secrete pro-inflammatory cytokines like tumour necrosis factor (TNFα), interleukin

(IL)-6 and IL-1β and acute phase proteins in serum, for example, amyloid A (Werling et al., 1996). Even though immune responses may not always protect the host (Medzhitov, 2007), they could be detrimental for growth or production performance of the host due to the high energy cost associated with stimulation of the immune system (Wolowczuk et al., 2008).

As demonstrated in **chapter 5**, the immune system of slower growing control chickens could be more active indicating stress as evidenced by higher relative abundance of genes in caecal bacteria encoding bacterial virulence factors; including biosynthesis of LPS (for example UDP-N-acetylglucosamine acyltransferase) and genes involved in the regulation of virulence; in control birds compared to the H57 treated birds. The presence of LPS in the gut could lead to translocation of this virulence factor into the portal system (Jacob et al., 1977), which even in minute amounts, could trigger an immune response (Bjorneboe et al., 1972, Van Leeuwen et al., 1994). Activation of the immune system by LPS, particularly an acute phase response, can significantly affect protein metabolism (Barnes et al., 2002), bone homeostasis and body composition in broiler chickens (Mireles et al., 2005). Several acute phase proteins are formed during immune challenge utilizing free amino acids in the blood (Barnes et al., 2002), which could otherwise be used for growth and production. Jiang and colleagues (Jiang et al., 2010) have shown that acute phase immune response artificially induced by LPS in chickens diverts a large proportion of feed energy from tissue accretion, resulting in depression of growth. Chickens need about 112 kcal/$kg^{0.75}$ of metabolisable energy (ME) per day for maintenance, about 10 kcal of ME/g for protein deposition and about 17 kcal of ME/g for fat deposition at 23°C with a quadratic effect of temperature on maintenance requirements (Sakomura et al., 2005). Therefore, interactions with the bird's immune system can affect energy partitioning with each 100 kcal of ME diverted towards immune system activation reducing body weight gain the equivalent to about 10 g of protein or about 6 g of fat. Moreover, immune system stimulation has also been found to reduce survival of birds (Hanssen et al., 2004). By contrast, translocation of LPS across the gut wall has been linked to weight gain in mice (Cani et al., 2007) and human beings (Creely et al., 2007). Further study is needed to explain this discrepancy between chickens and mammals.

A multi-strain probiotic containing *Enterococcus faecium, Lactobacillus reuteri, L. salivarius* and *Pediococcus acidilactici* partially alleviated the growth depression due to LPS induced immune response in broiler chickens (Jiang et al., 2010). Positive responses to H57 only occurred when there was depression of growth **(chapter 3)**, modulation of intestinal microbial profile by H57 **(chapter 4)** and evidence for a larger amount of virulence factors in control chickens when compared with H57 fed chickens **(chapter 5)**. This led to the presumption that H57 modulates the intestinal microbial structure or function to reduce microbial virulence factor(s) responsible for

depression of growth in chickens. However, the extent to which these virulence factors activate the immune response and thereby affect growth needs further study. Any effect on immune system activation in the host could be assessed by investigating the differences in immunity-related gene expression in the intestinal tissue of the host or level of immune system activation-related marker molecules in blood. Although there are strong indications that one of the probable mode of actions of H57 is by reducing microbial virulence factors in the gut thereby reducing the expenditure of energy on the immune system, variation of results between experiments and between diets also indicates that this is unlikely to be the sole mechanism.

LPS are structural components in outer membranes of the gram-negative bacteria. There was a higher abundance of LPS synthesis enzymes in the caeca of control chickens even without significant differences between microbial profiles of Control and H57 treatment groups in experiment 3 **(chapter 5).** In contrast to this link between LPS and growth rate in experiment 3, there was a substantial increase in the gram-negative *Bacteroides* population in the H57 group, as determined by 16S rRNA gene sequencing, when compared with the Controls in experiment 1 (**Chapter 4**). However, no metagenomic analysis was undertaken to assess the level of LPS synthesis genes in the caeca of birds in experiment 1. There was a positive response to H57 in both of the experiments. This indicates that there might be other modes of action of H57, in addition to the potential reduction in immune stimulating microbial virulence factors by H57.

The relative abundance of genes responsible for antibiotic resistance, Shiga-toxin clusters and other important virulence factors found in microbes, were also significantly reduced in the H57 group (**chapter 5**). The emergence of antibiotic resistant pathogens has been identified as a major public health threat in recent times (World Health Organization, 2014, Roca et al., 2015, Ventola, 2015). Therefore, this effect of H57 could represent an important benefit relating to public health and warrants closer investigation in future studies. Shiga-toxin producing *E. coli* (STEC), are important zoonotic pathogens causing diseases like haemorrhagic colitis and haemolitic uremic syndrome (HUS) which can result in acute kidney failure in children (Karmali et al., 2010, Gyles, 2007). As reducing the contamination of animal products, particularly meat, with STEC has been a major emphasis in the public health sector, the effect of H57 to reduce Shiga-toxin cluster genes is interesting to pursue in future studies.

7.2.2 Short chain fatty acids – important metabolites in the GIT affecting energy homeostasis

The suppression of growth of chickens also appeared to be associated with diet, as growth was depressed either with wheat or sorghum based diets but not with a mixed (wheat + sorghum) diet in experiment 3 (**chapter 3**). Also, growth depression and the effect of H57 varied with the experiment or batch/source of chicks as indicated by a significant difference in weight gain in experiment 1 and 3 but not in experiment 2. Intestinal microbial profiles were significantly different between the experiments. These differences may be the result of different 'seed microbes' colonising the GIT from the hatchery or rearing environment (feed, water, litter etc.) during the initial days of the chickens' life. Thus, the growth depression or the effect of H57 may be associated, at least in part, with the composition of the resident microbes in the gut.

The association of intestinal microbes with the suppression of growth in chickens has been established from past studies comparing growth of germ free birds with birds in a normal production environment (Forbes and Park, 1959, Forbes et al., 1959, Coates et al., 1963). Therefore, the apparent inconsistency in the effects of H57 reported in this thesis could be associated with different initial resident microbes in the gastrointestinal tract of chickens. Due to the apparent association between intestinal microbiota with growth rate and the beneficial effects of H57, modification of microbial function in the GIT by H57 is likely to have a subsequent effect on the energy homeostasis of the chickens. Based on past studies in mice and humans with host-microbes interactions, two of the most important metabolites in the intestine affecting energy homeostasis of the host are short chain fatty acids (SCFAs) (Byrne et al., 2015) and bile salts (Krogdahl, 1985, Maldonado-Valderrama et al., 2011).

Chickens cannot digest digestive-enzyme-resistant non-starch polysaccharides, and these carbohydrates are not digested in the small intestine, reaching the caeca undigested (Józefiak et al., 2004). The resident microbes in the caeca digest these carbohydrates through fermentation producing SCFA, mainly acetate, propionate and butyrate. However, the type and proportion of SCFA produced depends upon the diet, intestinal microbiota diversity and intestinal transit time (Brinkworth et al., 2009, Wisker et al., 1988, Peng et al., 2013, Murphy et al., 2010).

SCFAs play an important role in appetite regulation and energy homeostasis in mice (Byrne et al., 2015), thereby affecting body weight gain and adiposity (Ridaura et al., 2013, Liou et al., 2013). In general, in the mouse and human models, SCFAs reduce energy intake and increase energy expenditure through several interconnected mechanisms involving liver, adipose tissue, pancreas,

intestinal wall, skeletal tissue and nervous system (Figure 7-1) thereby reducing bodyweight (Byrne et al., 2015). Several studies, mainly on a mammalian (mouse) model, have shown how microbial SCFAs may regulate body weight in the host. Ridaura *et al.* (Ridaura et al., 2013) transplanted faecal microbiota of twin mice differing in body weight (obese vs lean) to germ free mice and showed that microbiota transplanted mice had similar body weight phenotype and microbiota to that of the donor. The amount of caecal propionate and butyrate was significantly lower in obese mice in comparison with their lean counterparts. Higher SCFA concentrations in lean mice were believed to inhibit fat accumulation. Similarly, Liou *et al.* (2013), showed that a faecal transplant from mice which had undergone bariatric (gastric bypass) surgery to germ free mice, increased the ratio of propionate to acetate, and this correlated with weight loss. It has been suggested that higher propionate levels inhibit the conversion of acetate into lipid in the liver and adipose tissue while reduced acetate production reduced lipogenesis (Hong et al., 2005, Wolever et al., 1991).

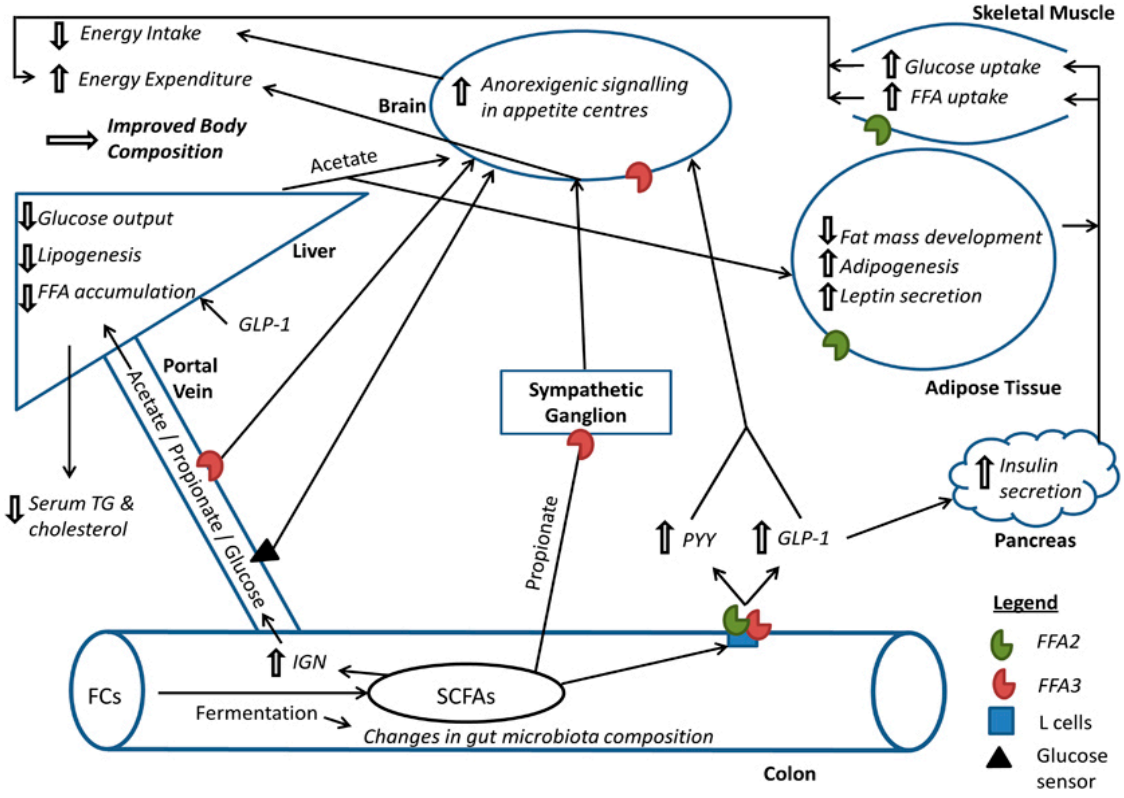


Figure 7-1 Mechanism of action of short chain fatty acids (SCFAS) to regulate appetite and energy homeostasis in the mice and human models. FCs, fermentable carbohydrates; FFA, free fatty acids; FFA2, free fatty acid receptor 2; FFA3, free fatty acid receptor 3; GLP-1, glucagon like peptide-1; IGN, intestinal gluconeogenesis; PYY, peptide YY; SCFAs, short chain fatty acids; TG, triglyceride. Diagram reproduced from Byrne *et al.* (2015).

No studies were uncovered about the role of SCFAs on feed intake and energy homeostasis in chickens. However, the relative abundance of genes encoding for fermentation was higher in H57 birds compared to control birds with a wheat based diet in experiment 3 (**chapter 5**). Therefore, fermentation of non-starch polysaccharides resulting in the production of SCFAs may have

different outcomes in chickens from that in mammalian (murine) model. Increased fermentation rate, as indicated by higher abundance of fermentation related genes in H57 treated birds, may be the indicator of higher microbial digestion of the non-starch polysaccharides which are regarded as a common anti-nutritional factor present in wheat resulting growth depression (Choct and Annison, 1990, Angkanaporn et al., 1994, Choct and Annison, 1992a, Choct and Annison, 1992b). The role of caecal microbes in digesting wheat pentosans was demonstrated by Choct *et al.* (Choct et al., 1992) using cecectomized broiler chickens. The faecal pentosan digestibility coefficient in cecectomized chickens was found to be significantly lower compared to that in normal chickens. Analysis of the transcripts from the genes related to fermentation expressed in the intestine, along with measurement of the quantity and composition of SCFAs production in the intestine, would help to further understand the role of fermentation and SCFAs in chicken metabolism.

7.2.3 Depression of growth by gut associated bile salt hydrolase activity

Bile acids (cholic acid and chenodeoxycholic acid) are important metabolites in the gut which can also act as hormones affecting energy homeostasis (Watanabe et al., 2006, Watanabe et al., 2012). Bile acids are mostly conjugated to glycine or taurine and exist in their anion form at physiological pH (as sodium salts, i.e. bile salts). While the role of bile salts in poultry has had little attention, studies in mammals showed that bile salts affect lipid, cholesterol and glucose metabolism (Thomas et al., 2008, Joyce and Gahan, 2016). Most of the bile salts in the intestine are directly recycled through uptake into the enterohepatic circulation (Small et al., 1972). Intestinal microbes and the host together determine the size of the bile salts pool (Ridlon et al., 2014). Bacterial bile salt hydrolase (BSH) activity removes glycine or taurine from conjugated bile acids forming their deconjugated counterparts (Figure 7-2), which are subsequently less efficiently recycled, affecting lipid and cholesterol metabolism and energy homeostasis (Joyce et al., 2014b, Joyce et al., 2014a).

Although the effect of BSH activity in poultry has not been studied extensively, intestinal microbes like *E. faecium* and *C. perfringens* with higher BSH activity have been shown to depress growth rates in chickens (Knarreborg et al., 2002a, Stutz and Lawton, 1984, Houghton et al., 1981). Deconjugated bile acids increased the metabolic rate in brown fat tissue resulting in weight loss in mice fed a high fat diet (Watanabe et al., 2006). When there is inefficient recycling of the bile salts leading to a reduced uptake of deconjugated bile salt uptake cf conjugated salts, this creates a relative energy deficit, which might have to be overcome by increased metabolism of brown fat leading to weight loss. It has been suggested that antibiotic growth promoters improve the growth rate of poultry by lowering BSH activity in the GI tract (Guban et al., 2006, Feighner and

Dashkevicz, 1988). In the context of antibiotic growth promoters being gradually phased out from animal diets, non-antibiotic BSH inhibitors could be promising growth promoters (Lin et al., 2014).

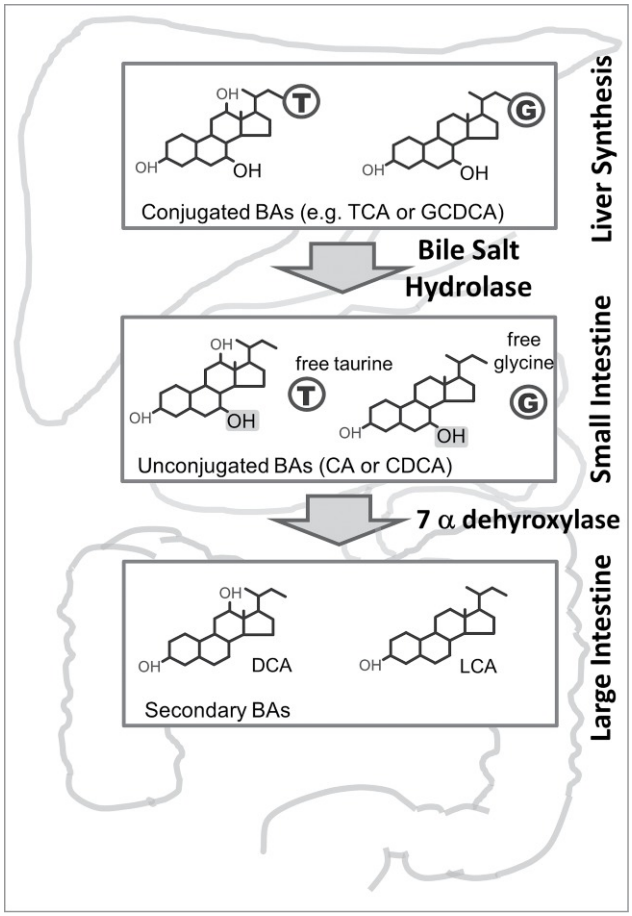


Figure 7-2 Metabolism of bile acids in the intestine. Picture copied from Joyce *et al.* (Joyce et al., 2014a). G = Glycine, T = Taurine, BAs = Bile Acids, CA = cholic acid, CDCA = chenodeoxycholic acid, TCA = taurocholic acid, GCDCA = glycochenodeoxycholic acid, DCA = deoxycholic acid, LCA = lithocholic acid. Conjugated bile salts are reabsorbed directly from the gut while the deconjugated salts are likely to be excreted.

It is postulated that modification of intestinal microbes by H57 might reduce the BSH activity in the intestine subsequently overcoming the depression of growth of the birds by reducing the uptake of deconjugated bile salts and thereby their effect on metabolic rate. While BSH is commonly produced by gut-associated bacteria (Jones et al., 2008), isozymes from different bacteria can have different activity due to differences in substrate specificity, potency and susceptibility to inhibitors. Moreover, microbially altered bile acids themselves can have direct or indirect effects on intestinal microbiota (Wahlström et al., 2016) amplifying the net effect. There was no effect of H57 on the relative abundance of BSH enzyme (choloylglycine hydrolase) encoding genes for the sorghum based diet in experiment 3. Unexpectedly, the relative abundance of BSH encoding genes was significantly higher in the H57 group when compared with the Control group fed a wheat based diet

in experiment 3. However, BSH activity could be of more significance in the small intestine than in the caecum. The abundance and expression of the BSH gene in experiments 1 and 2 and BSH expression in experiment 3 were not examined. Further studies are needed to determine if H57 has an effect on BSH activity in the chicken GIT particularly in the small intestine. Measurement of bile salts in digesta and serum samples together with transcriptome analysis to see the expression level of BSH gene in Control and H57 fed birds would be insightful complemented by a metabolomic study.

7.2.4 Feed intake and regulation of appetite in poultry

Increased growth rate in the H57 fed birds, particularly in experiment 3, was concurrent with a significant increase in feed intake compared to Control birds. Supressed appetite could be one of the factors causing lower growth rates in Control birds. However, higher feed intake might not be the sole cause of better performance of H57 groups as feed use efficiency was also improved by inclusion of H57 as a probiotic.

Feed intake in chickens is regulated through short-term (meal to meal intake) regulation by satiety signals between digestive system (intestinal tract, liver and pancreas) and the satiety centre in the brainstem and long term balance of energy storage by signalling between adipose tissue, liver and hypothalamus (Richards, 2003, Richards and Proszkowiec-Weglarz, 2007). Presence of feed and nutrients in the intestine releases cholecystokinin and bombesin, which inhibit feed intake by stimulating the satiety centre in the brainstem (Denbow, 1994, Kuenzel, 1994, Jensen, 2001). Ghrelin, a potent peptide responsible for appetite stimulation in mammals (Wren et al., 2000), also suppresses feed intake in chickens (Furuse et al., 2001, Saito et al., 2002). Long term energy balance in the body is maintained by regulation of feed intake and energy expenditure, mainly through the levels of leptin and insulin together with several other peptides (Richards and Proszkowiec-Weglarz, 2007, Richards, 2003). Leptin and its receptor play crucial roles for energy homeostasis and feed intake affecting body weight and accumulation of adipose tissue (Barb et al., 1998, Friedman and Halaas, 1998). Leptin inhibits feed intake (Barb et al., 1998). However, the presence of a leptin gene in chickens has been controversial for years with some recent evidence supporting the presence of a leptin gene in chickens (Seroussi et al., 2015, Farkašová et al., 2016). Further study is warranted on the relationship between feed intake and appetite regulation and the probiotic mode of action of H57. For this, measurement of hormone levels, other metabolites in blood and expression of appetite related genes following H57 administration would be the starting point.

7.3 Commercial potential for H57

There are strong indications that H57 can affect different microbiomes (**chapter 4**) and/or a range of microbial functions (**chapter 5**) in the broiler GIT. This is very important in the context of dynamic intestinal microbial populations whose constitution changes with age, diet, health condition etc. Positive responses to H57 with different sources of chicks, different gut microbial populations and different feed ingredients (wheat and sorghum) are positive outcomes for any future commercial developments.

A higher dose rate of H57 was used in experiment 3 than in experiments 1 and 2 to obtain a range of dose rates, at which H57 may show positive effects. This range could be used in the next phase of studies as a yardstick around which to establish dose related responses, and optimum dose rate. It would also help establish commercial fermentation population requirements leading to media optimisation and a fermentation method to produce the required spore to vegetative cell ratio which engenders the optimum growth responses in poultry. This will determine in part the prospects for commercial use of this bacterial strain as a probiotic.

Growth media constitutes about one third of the total cost of bacterial fermentation (Rodrigues et al., 2006). In these studies mostly pure chemicals were used in the growth medium, but other agricultural and industrial by-products could be tested in future studies to reduce the cost. Examples of such products include whey (Timmer and Kromkamp, 1994, Øyaas et al., 1996), molasses (Montelongo et al., 1993, Göksungur and Güvenç, 1997) starch (Xiaodong et al., 1997) and legume flour (Altaf et al., 2006). As well, bentonite was used as a carrier in the experiments reported in this thesis but H57 has been previously used as HayRite in a whey carrier. Methods and costs of drying the microbial cell paste concentrated following fermentation (by centrifugation, freeze drying or some other method) also remain to be determined. This process is where the mix of spore and vegetative cells required is going to be very important as some methods of drying require heat which may be detrimental to vegetative cell survival.

Results described in this thesis indicate that H57 has potential commercial prospects. Several lines of research are required to elucidate the mode of action leading to optimisation of conditions under which a growth response in poultry can be expected. Fermentation protocols to reproduce the response at a commercially acceptable cost need to be developed. The intellectual property around some of the results in this thesis has been protected by patent applications under examination in Australia, USA, Europe, and South East Asia.

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Appendix 1. Supplementary material for chapter 2

Probiotics in animal nutrition: production, impacts and regulation[‡‡]

Yadav S Bajagai, Athol V Klieve, Peter J Dart, Wayne L Bryden

1 Introduction

Please see Introduction (section 2.1) in chapter 2.

2 Probiotics: Definition and Classification

2.1 Definition

Please see Introduction (section 2.1) in chapter 2.

2.2 Classification

There is an array of microorganisms used as probiotics which can be classified as follows.

1. **Bacterial vs Non-bacterial probiotics:** With the exception of certain yeast and fungal probiotics most of the microorganisms used are bacteria. Examples: Bacterial probiotics – several species of *Lactobacillus* (Mookiah et al., 2014), *Bifidobacterium* (Pedroso et al., 2013, Khaksar et al., 2012), *Bacillus* (Abdelqader et al., 2013), and *Enterococcus* (Mountzouris et al., 2010); Non-bacterial (yeast or fungal) probiotics - *Aspergillus oryzae* (Daskiran et al., 2012, Shim et al., 2012), *Candida pintolopesii* (Daskiran et al., 2012), *Saccharomyces bourlardii*, (Rahman et al., 2013), and *Saccharomyces cerevisiae* (Bai et al., 2013).

2. **Spore forming vs Non-spore forming probiotics:** Although non spore forming *Lactobacillus* and *Bifidobacterium* strains predominated initially, spore forming bacteria are now used e.g. *Bacillus subtilis* (Alexopoulos et al., 2004a) and *Bacillus amyloliquefaciens* (Ahmed et al., 2014).

[‡‡] This literature review with wider scope covering impacts of probiotics in poultry, pig and ruminants; and safety and regulation of probiotics has been done during candidature of this PhD and published by Food and Agriculture Organization of the United Nations (Ed. Harinder P S Makkar) (http://www.fao.org/3/a-i5933e.pdf). Remaining sections of this publication is presented in Chapter 2.

3. **Multi-species (or strain) probiotics vs Single-species (or strain) probiotics:** The microbial composition of probiotic products ranges from a single strain to multistrain/species compositions (Table S1). Examples of multi-species probiotics are PoultryStar ME (contains *Enterococcus faecium*, *Lactobacillus reuteri*, *L. salivarius*, and *Pediococcus acidilactici*) (Giannenas et al., 2012), PrimaLac (contains *Lactobacillus* spp, *E. faecium*, and *Bifidobacterium thermophilum*) (Pedroso et al., 2013), and Microguard (contains different species of *Lactobacillus, Bacillus, Streptococcus, Bifidobacterium* and *Saccharomyces* (Rahman et al., 2013). Single-species probiotics include Bro-bio-fair (*Saccharomyces servisia*) (Abdel-Raheem et al., 2012) and Anta Pro EF (*E. faecium*) (Abdel-Rahman et al., 2013) .

4. **Allochthonous probiotics vs Autochthonous probiotics:** The microorganisms used as probiotics which are normally not present in the GIT of animals are referred to as allochthonous (eg yeasts), while the microorganisms normally present as indigenous inhabitants of the GIT are referred to as autochthonous probiotics (eg. *Lactobacillus* and *Bifidobacterium*).

3 Microorganisms used in probiotics

Many commercial products use multi-strain probiotics although the benefits of using more than one strain or species in a single product has not been clearly established (Zhao et al., 2013). Microorganisms that have been used as probiotics in animal feed are listed in Table S1.

Table S4: Microorganisms used as probiotics in animal diet

Genus	Species	Strains	Commercial products containing the species	References
Aspergillus	*oryzae*			(Daskiran et al., 2012, Shim et al., 2012)
	niger			(Seo et al., 2010)
Bacillus	*amyloliquefaciens*	CECT 5940	Ecobiol (Norel Animal Nutrition, Madrid, Spain)	(Ortiz et al., 2013)
	toyonensis	BCT-7112	Toyocerin (Rubinum S.A., Barcelona, Spain)	(Taras et al., 2005, Kantas et al., 2015)
	coagulans	ATCC 7050, ZJU0616	-	(Adami and Cavazzoni, 1999, Hung et al., 2012)
	licheniformis	DSM 5749	Microguard, (PeterLab Holdings, Negeri Sembilan, Malaysia), LSP 122 (Alpharma, Vega Baja, Puerto Rico), BioPlus 2B (Chr Hansen, Hørsholm, Denmark), Probios (Chr Hansen, Hørsholm, Denmark), BioPlus YC (Evonik Industries, Essen, Germany), Enhancer (Performance Plus, Chicago, IL, USA)	(Alexopoulos et al., 2004a, Rahman et al., 2013, Abdel-Hafeez et al., 2017)
	megaterium	-	Microguard, (PeterLab Holdings, Negeri Sembilan, Malaysia)	(Rahman et al., 2013)
	mesentricus	-	Microguard, (PeterLab Holdings, Negeri Sembilan, Malaysia)	(Rahman et al., 2013)
	polymyxa	-	Microguard, (PeterLab Holdings, Negeri Sembilan, Malaysia)	(Rahman et al., 2013)
	subtilis	588, CA #20, DSM 17299, PB6, ATCC-PTA 6737, DSM 5750	GalliPro (Evonik Industries, Essen, Germany), Microguard, (PeterLab Holdings, Negeri Sembilan, Malaysia), Super-CyC (Choong Ang Biotech Co. Ltd., Gyeonggy, South Korea), CloSTATTM (Kemin Industries Inc., Des Moines, USA), MicroSource "S" (Agtech Products Inc., Waukesha, USA), BioPlus 2B (Chr Hansen, Hørsholm, Denmark), Probios (Chr Hansen, Hørsholm, Denmark), BioPlus YC (Evonik Industries, Essen, Germany), Enviva Pro (DANISCO Animal Nutrition, Wiltshire, UK), Probion	(Alexopoulos et al., 2004a, Rahman et al., 2013, Afsharmanesh and Sadaghi, 2014, Davis et al., 2008, Abudabos et al., 2015, Abdel-Hafeez et al., 2017)

Genus	Species	Strains	Commercial products containing the species	References
			(Woogene B&G Co. Ltd., Seoul, South Korea), Enhancer (Performance Plus, Chicago, IL, USA)	
Brevibacillus	*laterosporus*	-	-	(Hashemzadeh et al., 2013)
Bifidobacterium	*animalis*	503, DSM 16284	PoultryStar ME (BIOMIN GmbH, Getzersdorf, Austria), Probios (Chr Hansen, Hørsholm, Denmark)	(Giannenas et al., 2012, Mountzouris et al., 2010, Wideman Jr et al., 2012)
	bifidium		PrimaLac (Star Labs, Inc., Clarksdale, USA), Protexin (International Animal Health Products, Huntingwood, Australia)	(Landy and Kavyani, 2013, Haghighi et al., 2008, Daskiran et al., 2012)
	bifidus		Microguard (PeterLab Holdings, Negeri Sembilan, Malaysia)	(Rahman et al., 2013)
	thermophilus		PrimaLac (Star Labs, Inc., Clarksdale, USA)	(Pedroso et al., 2013, Khaksar et al., 2012)
	longum			(Seo et al., 2010)
	pseudolongum			(Seo et al., 2010)
	lactis			(Seo et al., 2010)
Candida	*pintolepesii*		Protexin (Probiotics International Ltd., Lopen Head, Somerset, UK)	(Daskiran et al., 2012)
Clostridium	*butyricum*		Probion (Woogene B&G Co. Ltd., Seoul, South Korea)	(Zhao et al., 2013, Zhang et al., 2012, Zhang et al., 2014)
Escherichia	*coli*	Nissle 1917		(Hashemzadeh et al., 2013)

Genus	Species	Strains	Commercial products containing the species	References
Enterococcus	*faecium*	589, NCIMB 11181, E1708, DSM 10663, NCIMB 10415, DSM 16211, DSM 3530, HJEF005	All-Lac (Alltech Inc., Nicholasville, USA), PoultryStar ME (BIOMIN GmbH, Getzersdorf, Austria), PrimaLac (Star Labs, Inc., Clarksdale, USA), Protexin (International Animal Health Products, Huntingwood, Australia), Pro-Soluble (Probiotics International (Protexin) Ltd., Somerset, UK), Anta Pro EF (Dr. Eckel GmbH, Niederzissen, Germany), Biomin IMBO (BIOMIN GmbH, Getzersdorf, Austria), Probios (Chr Hansen, Hørsholm, Denmark) UltraCruz (Santa Cruz Animal Health, Paso Robles, USA)	(Pedroso et al., 2013, Chawla et al., 2013, Zhao et al., 2013, Wideman Jr et al., 2012, Landy and Kavyani, 2013, Giannenas et al., 2012, Abdel-Rahman et al., 2013, Mountzouris et al., 2010, Cao et al., 2013, Khaksar et al., 2012)
	faecalis			(Seo et al., 2010)
Lactobacillus	*thermophilus*		All-Lac (Alltech Inc., Nicholasville, USA)	(Pedroso et al., 2013)
	acidophilus		Probios (Chr Hansen, Hørsholm, Denmark), Microguard (PeterLab Holdings, Negeri Sembilan, Malaysia), Protexin (International Animal Health Products, Huntingwood Australia), UltraCruz (Santa Cruz Animal Health, Paso Robles, USA), PrimaLac, Avian PAC Soluble, Probion (Woogene B&G Co. Ltd., Seoul, South Korea)	(Haghighi et al., 2008, Zhang et al., 2014, Rahman et al., 2013, Daskiran et al., 2012, Khaksar et al., 2012, Shim et al., 2012, Morishita et al., 1997)
	brevis	I 12, I 211, I 218, I 23, I 25		(Mookiah et al., 2014)
	bulgaricus		Microguard, (PeterLab Holdings, Negeri Sembilan, Malaysia), Protexin (International Animal Health Products, Huntingwood, Australia)	(Rahman et al., 2013, Daskiran et al., 2012)
	casei	CECT 4043	PrimaLac (Star Labs, Inc., Clarksdale, USA), , Probios, UltraCruz (Santa Cruz Animal Health, Paso Robles, USA)	(Khaksar et al., 2012, Landy and Kavyani, 2013, Fajardo et al., 2012)
	delbrueckii subspecies *bulgaricus*		Protexin (International Animal Health Products, Huntingwood, Australia)	(Daskiran et al., 2012)

Genus	Species	Strains	Commercial products containing the species	References
	farciminis		Enviva MPI (DANISCO Animal Nutrition, Wiltshire, UK)	
	fermentum	JS	JSA-101 Gold (Well-being LS Co. Ltd., Gangwon, Korea)	(Bai et al., 2013)
	gallinarum	I 16, I 26, LCB 12		(Mookiah et al., 2014, Ohya et al., 2000)
	jensenii			(Sato et al., 2009)
	paracasei			(Bomba et al., 2002)
	plantarum		Microguard (PeterLab Holdings, Negeri Sembilan, Malaysia), Protexin (International Animal Health Products, Huntingwood, Australia), UltraCruz (Santa Cruz Animal Health, Paso Robles, USA), Probios (Chr Hansen, Hørsholm, Denmark)	(Rahman et al., 2013, Daskiran et al., 2012, Peng et al., 2016)
	reuteri	514, C 1, C10, C16, DSM 16350, DSM 16350	PoultryStar ME (BIOMIN GmbH, Getzersdorf, Austria)	(Giannenas et al., 2012, Mookiah et al., 2014, Mountzouris et al., 2010, Wideman Jr et al., 2012)
	rhamnosus		Protexin (International Animal Health Products, Huntingwood, Australia), Enviva MPI (DANISCO Animal Nutrition, Wiltshire, UK)	(Daskiran et al., 2012, Hashemzadeh et al., 2013)
	lactis		Probios (Chr Hansen, Hørsholm, Denmark)	
	salivarius	DSM 16351, I 24	FloraMax-B11 (Pacific Vet Group, Fayetteville , USA), PoultryStar ME (BIOMIN GmbH, Getzersdorf, Austria)	(Biloni et al., 2013, Mookiah et al., 2014, Mountzouris et al., 2010)
	sobrius			(Konstantinov et al., 2008)
Lactococcus	*lactis*	CECT 539		(Fajardo et al., 2012)

Genus	Species	Strains	Commercial products containing the species	References
Megasphaera	*elsdenii*			(Seo et al., 2010)
Pediococcus	*acidilactici*	DSM 16210	All-Lac (Alltech Inc., Nicholasville, USA), PoultryStar ME (BIOMIN GmbH, Getzersdorf, Austria)	(Pedroso et al., 2013, Mountzouris et al., 2010, Wideman Jr et al., 2012)
	parvulus		FloraMax-B11 (Pacific Vet Group, Fayetteville, USA)	(Biloni et al., 2013)
Prevotella	*bryantii*			(Seo et al., 2010)
Propionibacterium	*shermanii*			(Seo et al., 2010)
	freudenreichii			(Seo et al., 2010)
	acidipropionici			(Seo et al., 2010)
	jensenii			(Seo et al., 2010)
Saccharomyces	*bourlrdii*		Microguard, (PeterLab Holdings, Negeri Sembilan, Malaysia)	(Rahman et al., 2013)
	cerevisiae	KCTC No.7193	JSA-101 Gold, Super-CyC (Choong Ang Biotech Co. Ltd., Gyeonggi, South Korea)	(Bai et al., 2013, Shim et al., 2012, Abdel-Rahman et al., 2013)
	servisia		Bro-biofair (Vitality Co., Egypt)	(Abdel-Raheem et al., 2012)
Streptococcus	*faecalis*			(Haghighi et al., 2008)
	faecium		Microguard (PeterLab Holdings, Negeri Sembilan, Malaysia), Avian PAC Soluble (Loveland Industries Inc., Colorado, USA)	(Rahman et al., 2013, Morishita et al., 1997)
	gallolyticus	TDGB 406		(Kumar et al., 2014)
	salivarius ssp. thermophilus		Protexin (International Animal Health Products, Huntingwood, Australia)	(Daskiran et al., 2012)
	bovis			(Seo et al., 2010)

4 Manufacture of probiotics

4.1 Selection of microbial strains

In addition to being non-pathogenic to animals, microorganisms used as probiotics are selected on the basis of their survival in the gastrointestinal environment and ability to withstand low pH and high concentrations of bile acids. In addition, the chosen strain should tolerate the manufacturing, transportation, storage and application processes, maintaining its viability and desirable characteristics (Collins et al., 1998). The capacity of potential probiotic microorganisms to withstand the gastrointestinal environment can be tested *in vitro* by challenging with low pH (Hood and Zoitola, 1988, Collado and Sanz, 2006). The capacity to tolerate an acidic environment and bile varies between strains (Mishra and Prasad, 2005). Another desirable characteristic is the ability to adhere to the intestinal epithelium, enabling the probiotic strain(s) to colonize the intestine (Guarner and Schaafsma, 1998). In addition, ability to grow rapidly on inexpensive media is a requisite (Collins et al., 1998) for economically viable production.

Spore forming bacteria, particularly from the genus *Bacillus*, are increasingly being used as probiotics. *Bacillus* spores are resistant to physical and environmental factors, such as heat, desiccation and UV radiation (Setlow, 2006, Mason and Setlow, 1986, Nicholson et al., 2000, Cutting, 2011) enabling them to maintain their viability during feed pelleting, storage and handling. *Bacillus lavolacticus* DSM 6475, and two species (total four strains) of *Sporolactobacillus* (*Sp. Inulinus* and *Sp. laevus*) were resistant to pH 3 and *B. racemilacticus* and *B. coagulans* were tolerant of bile (Hyronimus et al., 2000).

4.2 Fermentation

Fermentation techniques are used either to produce microbial cells in large quantity or to produce extracellular microbial products (e.g. food-grade lactic acid), enzymes, amino acids, vitamins and other pharmaceutical compounds.

Animal studies have used probiotics cultured in the laboratory (Zhou et al., 2010, Shim et al., 2012), or commercially available probiotics. Upscaling from the laboratory to a commercial product is not a trivial process and quality control is paramount for a beneficial product outcome.

4.2.1 Growth media

Microorganism specific growth media, either synthetic or dairy based, are generally used to grow probiotics in an economically viable way (Muller et al., 2009). Approximately 30% of the total cost of fermentation is media cost (Rodrigues et al., 2006). Dairy based media have been preferred for production of human probiotics, with the use of dairy based foods such as yoghurt as the carrier. Some countries have legal requirements preventing the use of synthetic media for the production of human probiotics (Muller et al., 2009), but there are no such restrictions for fermentation media for the production of probiotics for animal use.

Use of pure chemical substrates as carbon sources (Javanainen and Linko, 1995, Xiaodong et al., 1997) for fermentation generally results in high quality products. However, agricultural and other industrial by-products are preferred substrates for fermentation because of reduced cost (Hofvendahl and Hahn–Hägerdal, 2000). For example, whey (Timmer and Kromkamp, 1994, Øyaas et al., 1996), molasses (Montelongo et al., 1993, Göksungur and Güvenç, 1997) and starch (Xiaodong et al., 1997) are popular substrates for industrial fermentation. Similarly, yeast extract and peptone are popular nitrogen sources in fermentation media (Chiarini et al., 1992). Yeast extract can be replaced with cheaper agricultural products (e.g. lentil flour) as nitrogen sources (Altaf et al., 2006). Feed grade vegetable proteins and food grade carbohydrates have also been used for production of commercial probiotics (European Food Safety Authority, 2008). However, media information is not available for most commercial probiotics.

The ideal growth medium that maximises microbial growth can be very complex and expensive (Muller et al., 2009). Different probiotic strains generally require different media.

4.2.2 Growth conditions

Temperature and pH affect fermentation growth rates, which are species and strain dependent. Optimum temperatures for *Lactobacillus* strains varies between 25^{o}C and 45^{o}C (Hofvendahl and Hahn–Hägerdal, 2000). Similarly, optimal pH for the growth of probiotics also varies with microbial species and strain. In some cases, pH is set at the beginning of fermentation and allowed to drift (often decreasing due to the production of acids) while fermentation proceeds while in other cases pH is kept fairly constant by adding buffer (Hofvendahl and Hahn–Hägerdal, 2000, Muller et al., 2009).

4.2.3 Fermentation methods

Probiotics can be produced by either batch or continuous fermentation. In the batch fermentation, all of the substrates (sterilized) and the inoculum are mixed together in the fermenter at the beginning and kept at the optimum temperature for the growth of the probiotic. In fed-batch fermentation limiting nutrients can be added during the fermentation. The reduction of pH in the fermentation medium, to the level where it inhibits the rate of microbial growth, is one of the challenges with batch fermentation and is generally managed by adding a base or a buffer to the medium to maintain pH (Muller et al., 2009). After completion of the fermentation process, which is generally determined by measuring the concentration of probiotic in the fermenter, cells are recovered by centrifugation or filtration (Champagne et al., 2007). Obtaining a high cellular concentration while maintaining low viscosity is an important objective in optimizing the batch fermentation process, as high viscosity hinders the recovery of cells from the growth medium (Champagne et al., 2007). For spore-forming bacteria, vegetative cells are induced to sporulate, generally by limiting nutrient availability, before harvesting. Adjustment of pH is another method of triggering sporulation.

With continuous fermentation, fresh growth medium is continuously added to the culture while bacterial cells and any inhibitory substances produced during fermentation are continuously removed so that continuous production of the probiotic can be maintained (Lamboley et al., 1997, Muller et al., 2009). Genetic drifts from mutation(s) or contamination with other bacteria occurring during the fermentation process are issues with continuous fermentation. Batch fermentation has been preferred because it is less costly than continuous fermentation (Muller et al., 2009).

Doleyres et al. (2004) developed a two-stage fermentation system as used in yoghurt production in a laboratory trial where the inoculum strain(s) was immobilised as a pure culture in carrageenan/locust bean gel beads which then released bacteria at a controlled rate into the linked, continuous fermentation reactor to produce probiotics containing the required ratio of *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* MD and *B. longum* ATCC 15707 cells but this ratio could not be maintained.

4.3 Drying

After fermentation the bacterial and yeast cells are usually dried for ease of transport and storage rather than needing the specialized facilities for storage and transport of liquid inoculants or frozen cells. Probiotic microorganisms are generally dried by freeze drying or spray drying (Muller et al.,

2009), but vacuum drying and fluidized bed drying are also used. Maintaining cell viability during drying is critical for successful probiotic production (Meng et al., 2008).

4.3.1 Freeze drying

A two-step process of freezing and drying is used. The bacteria are first frozen by using liquid nitrogen or dry ice or refrigerated at -20^0 and then dried under high vacuum to reduce the moisture level to 4% or below (Ananta et al., 2004). During freezing, the process should be fast enough to prevent the formation of ice crystals inside the cell (Mazur, 1976). Although this is the best method to dry bacteria, in terms of maintaining viability, the high cost associated with the process often hinders its application (Chávez and Ledeboer, 2007).

Similarly, yeast cultures have also been preserved and stored by freeze drying (Kawamura et al., 1995). A modification of the standard freeze drying method involving evaporative cooling can preserve yeast cells for 30 years (Bond, 2007). In this method, a centrifugal head connected with a freeze dryer is used to initially dry the yeast culture mixed with lyoprotectant followed by secondary drying under vacuum using phosphorus pentoxide as a desiccant. Dehydration of yeast cells with successive reduction in pressure is a feasible alternative to freeze-drying (Rakotozafy et al., 2000).

4.3.2 Spray drying

Fine droplets of probiotic culture, atomized by spraying through a heated nozzle, are sprayed into the drying chamber against hot air (Knorr, 1998, Masters, 1972). The microorganisms (bacteria or yeast) are dried during the process and collected at the bottom of the chamber (Masters, 1972). The exposure to the high temperature during drying can kill a significant proportion of the vegetative cells, a major limitation (Elizondo and Labuza, 1974). However, the technique is popular because of the low cost of drying for the bulk production of probiotics. It is more suitable for drying spores as the probiotic product.

Probiotic microorganisms are generally produced by a fermentation process with species and strain specific temperature and pH and mostly dried by a freeze-drying or spray drying process. Growth in inexpensive media is important for commercial production. Probiotics for animal nutrition need to maintain their viability during manufacturing, storage and handling and quality control is needed to ensure this. Probiotics are selected to presumably withstand the gastrointestinal environment and adhere to the intestinal epithelium.

5 Mode of probiotic action

Please see section 2.3 in chapter 2.

6 Probiotic application in different livestock production systems

6.1 Probiotics in poultry nutrition

Please see section 2.4 in chapter 2.

6.2 Probiotics in pig nutrition

Although banned in some countries including the EU, sub-therapeutic use of antibiotics in feed to prevent diarrhoea and improve performance is still common in the swine industry. Therefore, the substitution of probiotics to address the issue of antibiotic resistance is critical in pig production and reduction in the use of antibiotics in feed. For monogastrics, this substitution has been more extensively studied in poultry than in pigs.

As with other livestock, it is difficult to make generalisations because of the variation in the microorganisms used, doses, duration of treatment and husbandry practices (Kenny et al., 2011).

6.2.1 Growth rate and feed use efficiency

Several probiotics have been used to enhance the performance of pigs (Table S2). In a large scale experiment in a high performing commercial setting, the commercial probiotic product BioPlus 2B containing *B. subtilis* and *B. licheniformis* was a viable substitute for AGPs (neomycin, oxytetracycline, tylosine etc.) without a decrease in weaned pig performance; and with no increase in production costs (Kritas and Morrison, 2005). BioPlus 2B also improved weight gain by up to 8% and feed use efficiency by up to 10% in grower and finisher pigs in a dose dependent manner (Alexopoulos et al., 2004b). For doses of 0.64, 1.28 and 1.92 × 10^6 cfu/g of feed, the daily gain increased with dose rate. Guo et al. (2006) also found *B. subtilis* MA139 effective in significantly improving FCR. Kyriakis et al. (1999) reported that average daily gain in post weaning piglets was increased by 99% over a period of 28 days when the piglets diet was supplemented with spores of *B. licheniformis* at the rate of 10^7 spores per gram of feed. Feed use efficiency was improved by 24%. In a recent study, the commercial probiotic product Toyocerin containing *Bacillus toyonensis* given to post-weaning piglets at the rate of 1.24x10^6 cfu per gram of feed improved average daily

gain by 5% over the 42 day experimental period (Kantas et al., 2015). Average daily feed intake was increased by 1.7% and feed use efficiency was improved by 4.7% over the same period. In contrast, another commercial probiotic product MicroSource "S," (Agtech Products Inc.) containing *B. licheniformis* and *B. subtilis* did not improve growth rate or feed intake (Davis et al., 2008) when fed at the very high dose rate of 1.47×10^8 cfu/g feed but did significantly improve feed use efficiency by 3%.

Supplementation of weaned pigs with 2×10^9 cfu/kg feed with *S. cerevisiae* sub. *boulardii* CNCM I-1079 for 6 weeks followed by 1×10^9cfu/kg feed of *P. acidilactici* CNCM MA 18/5 M for 3weeks significantly improved the FCR without affecting intestinal structure (villus height, crypt depth, the goblet cell number and the thickness of the mucus layer) (Le Bon et al., 2010). In contrast, Van Heugten et al. (2003) did not observe any positive responses in growth or nutrient digestibility when *S. cerevisiae* SC47 was added to a pig diet at a rate of 1.6×10^7 cfu/g of feed.

Table S5: Probiotic effects on performance of pigs

Microorganisms	Growth rate (ADG)	FCR	Feed intake	Age group	Reference
B. subtilis and *C. butyricum*	S (+)	S (-)	NS	Growing-finishing pigs	(Meng et al., 2010)
L. acidophilus, S. cerevisae and B. subtilis	S (+)	NS	NS	Growing pigs	(Chen et al., 2005)
L. plantarum ATCC 4336, *L. fermentum* DSM 20016 and *E. faecium* ATCC 19434	S (+)	NS	-	Weaned piglets	(Veizaj-Delia et al., 2010)
E. faecium EK13	NS	-	-	Newborn piglets	(Strompfova et al., 2006)
Bi. longum (AH1206)	NS	NS	-	Neonatal piglets	(Herfel et al., 2013)
B. licheniformis	S (+)	S (-)	-	Weaned piglets	(Kyriakis et al., 1999)
B. subtilis and *B. licheniformis*	S (+)	S (-)	NS	Growing pigs	(Kritas et al., 2000)
B. subtilis and *B.*	S (+)	S (-)	NS	Grower	(Alexopoulos et al., 2004b)

licheniformis				finisher pigs	
B. subtilis MA139	NS	S (-)	NS	Weaned piglets	(Guo et al., 2006)
Bacillus toyonensis	S (+)	S (-)	S (+)	Weaning piglets	(Kantas et al., 2015)
B. licheniformis and *B. subtilis*	NS	S (-)	NS	Growing-finishing pigs	(Davis et al., 2008)
S. cerevisiae sub. *boulardii* CNCM I-1079	-	S (-)	-	Weaned piglets	(Le Bon et al., 2010)

S (+) = significantly increased, S (-) = significantly decreased, NS = non-significant, - = not studied, ADG = average daily gain, FCR = feed conversion ratio

Similarly, probiotic *L. sobrius* DSM 16698 was effective in improving average daily gain by 74% with 6% increase in feed intake in piglets infected with enterotoxigenic *E. coli* and also fed the probiotic at the high rate of 10^{10} cfu/animal/day (Konstantinov et al., 2008). In another experiment, final body weight was not improved when *L. amylovorus* and *E. faecium* were fed at the rate of $3x10^{8}$ cfu/animal/day (Ross et al., 2010). However feed intake was significantly reduced with improvement in feed use efficiency by 15% to 42% during different periods of the experiment. Likewise, application of *E. faecium* to primiparous sows at 5×10^{8} cfu/kg feed, increased feed intake and improved reproductive performance (Böhmer et al., 2006).

Use of different strains and doses of microorganisms and differences in husbandry practices (nutrition, housing etc.), and age of pigs and feed type may explain contrasting results with the same species of probiotic microorganisms.

Probiotics can enhance the growth of pig but with less consistent results than for poultry.

6.2.2 Health

Adding the commercial probiotic containing *B. licheniformis* and *B. subtilis* spores (BioPlus 2B) to the diet of weaned, grower and finisher pigs at the rate of $0.64 – 1.28 \times 10^{6}$ cfu/g feed significantly reduced morbidity and mortality (Alexopoulos et al., 2004b). The same combination of probiotics when fed to pregnant sows from two weeks prior to expected farrowing date and during lactation improved the performance of the litter with reduced piglet diarrhoea, reduced pre-weaning mortality and increased body weight at weaning (Alexopoulos et al., 2004a). Decreased weight loss in sows during lactation and production of milk with higher fat and protein content were suggested reasons for the improved health and performance of the piglets.

Probiotics inhibits the adhesion of enteric pathogens in intestinal mucosa. *Bi. lactis* Bb12 and *L. rhamnosus* LGG individually or in combination inhibited adhesion of pathogens (*Salmonella, Clostridium,* and *E. coli*) to the intestinal mucosa collected from young healthy pigs in an *in vitro* experiment (Collado et al., 2007). Adhesion of pathogens was measured by using radioactively labelled microorganisms and measuring radioactivity before and after adhesion to the intestinal mucosa. However, Szabo et al. (2009) found that *E. faecium* NCIMB 10415 treatment did not improve the clinical signs in pigs experimentally infected with *S. enterica* serovar *typhimurium* DT104.

Post weaning diarrhoea, caused mainly by enterotoxigenic *E. coli*, is one of the major health problems in swine worldwide causing substantial economic losses due to mortality, reduced growth rate and associated veterinary costs (Fairbrother et al., 2005). Probiotics reduced the incidence and severity of post weaning diarrhoea in pigs. Supplementation of weaned piglet diets with *B. licheniformis* spores at the rate of 10^6 and 10^7 cfu/g of feed significantly reduced post weaning diarrhoea and associated mortality (Kyriakis et al., 1999). Performance of piglets fed the higher dose (10^7 cfu/g) of probiotics was better than those fed the lower dose. In another study, the incidence of post-weaning diarrhoea decreased following the addition of *B. toyonensis* to the diet of pregnant sows from 90 days before farrowing to 28 days *postpartum* and in the diet of piglets from days 15 to 56 (Taras et al., 2005). Kantas *et al.* (2015) also demonstrated the beneficial effects of *B. toyonensis* (commercialised as Toyocerin) to reduce the enteric pathogen load and diarrhoea in post weaning piglets.

Probiotics reduced intestinal colonization by pathogenic *E. coli* and prevented or reduced the severity of the intestinal infection. The level of enterotoxigenic *E. coli* in the ileum of experimentally infected piglets after weaning was significantly lowered by treating with *L. sobrius.* (Konstantinov et al., 2008). *L. paracasei* mixed with maltodextrin also reduced intestinal colonization by *E. coli* in piglets raised in an apparently sterile environment (Bomba et al., 2002). Similarly, translocation of pathogenic *E. coli* to mesenteric lymph nodes was reduced in pigs treated with *P. acidilactici* and *S. cerevisiae ssp. boulardii* and then challenged with pathogenic *E. coli* (Lessard et al., 2009). Positive effects on intestinal barrier function may be the possible mode of action for these probiotic effects.

Le Bon et al. (2010) found a dramatic reduction in the level of *E. coli* after four weeks of treatment with *S. boulardii* and *P. acidilactici* in weaned piglets. Similarly, *E. faecium* added to pig diets controlled post weaning diarrhoea and mortality due to *E. coli* infection (Underdahl et al., 1982, Taras et al., 2006, Zeyner and Boldt, 2006).

Probiotics can be effective in reducing post weaning diarrhoea in piglets and morbidity and mortality in pigs.

6.2.3 GIT microbial population

A single large dose ($5x10^9$ or $5x10^{10}$) of *L. plantarum* (DSMZ 8862 and 8866) given to piglets one week before weaning or at weaning resulted in a significant change in the microbial population of the small and large intestines (Pieper et al., 2009). However, the observations were only made at 2 weeks post treatment and did not explore the long-term effects of the single administration. In another study, the probiotic *L. paracasei* mixed with fructo-oligosaccharides increased populations of *Lactobacillus* spp., *Bifidobacterium* spp., total anaerobes and total aerobes and decreased *Clostridium* and *Enterobacterium* in faeces of weanling pigs (Bomba et al., 2002). Similarly, *S. cerevisiae* and *P. acidilactici* produced a temporary (about two weeks) reduction in the population of *E. coli* and other coliforms in pig faeces after application of probiotics for four weeks at 2×10^9 cfu/kg feed (Le Bon et al., 2010). However in other trials, inclusion of a yeast probiotic (*S. cerevisiae*) did not change the populations of *E. coli*, *Streptococcus*, *Lactobacillus* and total culturable yeast in the GIT, as it did in some earlier feeding trials (Mathew et al., 1998, Li et al., 2006). Nevertheless, pigs fed the probiotics performed better in terms of body weight gain and feed use efficiency in these experiments. Enhancement in performance in probiotic fed animals is apparently not necessarily associated with a change in the gastrointestinal microbial population that can be cultured. However, sequencing of the GIT microbial DNA indicates that the microbiome diversity is dominated by microbial species that have not yet been cultured.

In pigs probiotics increased lactic acid bacteria and decreased Clostridium, E. coli *and* Enterobacterium spp. *in the GIT.*

6.3 Probiotics in ruminant nutrition

The rumen has a complex microbial ecology where polysaccharides and protein ingested by the host are degraded by rumen microorganisms resulting in the synthesis of SCFA and microbial protein which are used by the host as energy and protein sources. There is increasing international interest in manipulating the rumen ecosystem to increase the efficiency of the ruminal fermentation processes to improve animal productivity and reduce unwanted by-products like methane.

Yeast (*S. cerevisiae*) is a commonly used probiotic in ruminants (Chaucheyras-Durand et al., 2008) affecting mainly the microbial population dynamics in the rumen and the breakdown of nutrients. Lactic acid producing bacteria are another important group of probiotics.

Apart from the use of probiotics in formulated animal feed, beneficial bacteria used as silage inoculants may also have a probiotic effects in the rumen (Weinberg et al., 2004). However, this response depends on the survival of the silage inoculant in the silage as the pH drops.

6.3.1 Milk yield

Probiotics can improve the milk yield in dairy animals. Milk yield was increased by 2.3 litre per cow per day following dietary supplementation with 5×10^9 cfu of *E. faecium* and 2×10^9 yeast cells (*S. cerevisiae*) per cow per day (Nocek and Kautz, 2006). Weiss et al. (2008) found that dairy cattle fed the probiotic *Propionibacterium* strain P169 had the same milk production as control animals but with decreased feed consumption, resulting in 4.4% increase in energy efficiency. Dietary supplementation with a combination of *L. acidophilus* NP51 and *P. freudenreichii* NP24 (4×10^9 cfu/animal/day) resulted in a 7.6% increase in average daily milk yield in Holstein cows (Boyd et al., 2011). Average milk yield per day increased by *c.*14% compared to non-treated, lactating Saanen dairy goats receiving *S. cerevisiae* at the rate of 4×10^9 cfu/day/animal (Stella et al., 2007).

Desnoyers et al. (2009) undertook a quantitative meta-analysis of 110 papers, 157 experiments and 376 treatments, studying the effects of yeast probiotics (containing at least one strain of *S. cerevisiae*) in ruminants (cattle, goats, sheep, and buffaloes) on feed intake, milk production and rumen fermentation. Supplementation with live yeast probiotics increased milk yield by about 1.2 g/kg body weight. Dry matter intake by the animals was increased by 0.44 g/kg of body weight. Overall the effect on milk yield was significant, but the results were highly variable and the economic benefits were not analyzed. There was no effect on milk protein content. A similar meta-analysis by Poppy *et al.* (2012) concluded that commercial probiotics containing *S. cerevisiae* increased milk yield by 1.18 kg/d, fat-corrected milk by 1.61 kg/d and energy-corrected milk by 1.65 kg/d. Similarly, dietary supplementation of *S. cerevisiae* increased milk fat yield by 0.06 kg/d and milk protein yield by 0.03 kg/d. Dry matter intake was increased by 0.62 kg/d during early lactation and 0.78 kg/d during late lactation. Increased feed intake together with improved microbial digestion (also see section 7.3.3) of feed could be the possible mode of action for improved animal performance.

In contrast, Krishnamoorthy and Krishnappa (1996) did not find any differences in dry matter intake, body weight gain, milk yield and milk composition when yeast was added in the diet based on finger millet (*Eleusine coracana*) straw of lactating crossbred cattle.

6.3.2 Growth

Probiotics can increase the weight gain of ruminants. For example, a probiotic containing a mixture of microorganisms (*L. reuteri* DDL 19, *L. alimentarius* DDL 48, *E. faecium* DDE 39 and *Bi. bifidium* DDBA) isolated from a healthy goat, when fed to goats for eight weeks, commencing at 75 days of age, resulted in improvement in average body weight by 9% (Apás et al., 2010). Similar improved growth rate was obtained with a yeast-based commercial probiotic containing *S. cerevisiae* given to growing dairy heifers (Ghazanfar et al., 2015). *B. amyloliquefaciens* strain H57 when fed to pregnant White Dorper ewes on a palm kernel based diet, increased DM intake and live weight gain during pregnancy followed by better performance of the lambs during early lactation (Le et al., 2014, McNeill et al., 2016). The same strain of *B. amyloliquefaciens* when fed to dairy calves at the rate of 3.16 x 10^8 cfu per kg dietary DM from week 4 to 12 improved growth rate by 39% (551 vs 767 g/d), increased feed use efficiency by 14% (2.5 vs 2.9 kg milk + starter DM/kg weight gain) (Le et al., 2016). Likewise, a novel bacterial strain isolated in Australia, *P. jensenii* 702, significantly enhanced weight gain in Holstein calves by 25% during pre-weaning period and by 50% during the weaning period (Adams et al., 2008).

Frizzo *et al.* (2011), based on meta-analysis of 21 publications between 1985 and 2010, concluded that lactic acid probiotic bacteria in comparisons with and without *L. acidophilus; L. plantarum; L. salivarius*; *E. faecium*; *L. casei/paracasei*; *Bifidobacterium spp*., increased body weight gain (standardized mean difference = 0.22822, 95% confidence interval = 0.1006 to 0.4638) and improved feed use efficiency (standardized mean difference = −0.8141, 95% CI = −1.2222 to −0.4059) in young calves compared to control groups when probiotics were added to milk replacer but were ineffective when added to whole milk. In contrast, some studies have reported no effect on calf growth when the diet was supplemented with *L. acidophilus* (Cruywagen et al., 1996, Abu-Tarboush et al., 1996), mixture of *L. acidophilus* and *Streptococcus faecium* (Higginbotham and Bath, 1993), mixture of *L. acidophilus* and *L. plantarum* (Abu-Tarboush et al., 1996), *B. subtilis* (Galina et al., 2009), mixture of *L. acidohilus, L. lactis*, and *B. subtilis* (Galina et al., 2009).

Quality control of the probiotics strain production and subsequent shelf viability is a critical component of trials assessing the affect they have when fed and often in nutrition trials this is inadequately dealt with and could be a reason for the variability in animal response between trials.

6.3.3 Nutrient digestibility

The improvement in performance by ruminants is often associated (at least partially) with improvement in nutrient digestibility. A combination of *L. acidophilus* NP51 and *P. freudenreichii*

NP24 improved the digestibility of crude protein, neutral detergent fiber and acid detergent fiber in lactating Holstein cows resulting in increased milk production per day by 7.6% without increase in dry matter intake (DMI) (Boyd et al., 2011) suggested to be due to a change in the rumen microbial ecosystem.. Similarly, supplementation of dairy cows with Probios TC containing 2 strains of *Enterococcus faecium* at the rate of $5x10^9$ cfu per day as well as $2x10^9$ viable yeast cells per day from 21 days prior to expected calving date through 10 week postpartum, increased milk production by 2.3 kg per cow per day, with no difference in 3.5% fat corrected milk. The *E. faecium* strains were thought to act by producing lactic acid which supported a rumen microbial population which increased ruminal digestion of roughages in the corn silage and haylage diet as well as increasing DMI (Nocek and Kautz, 2006). In contrast, Hristov et al. (2010) did not find any improvement in digestibility of corn silage based diet from supplementation with a yeast (*S. cerevisiae*) probiotic in Holstein cows. Although the yeast supplementation increased ruminal microbial protein synthesis, there was no difference in dry matter intake, milk yield and milk composition.

Based on a meta-analysis of papers published on the effects of yeast probiotics in all ruminant species reared for milk or meat, Desnoyers et al. (2009) found much variability in response with an overall average increase in DM intake by 0.44 g/kg body weight and total tract organic matter digestibility by 0.8%, effects too small to warrant probiotic addition. However particular strains, increasing levels of inoculum addition, and feed compositions with a larger proportion of concentrates have produced a better response than this average. Improvement in microbial digestion of feed may be either due to production of enzymes by probiotics or alterations in rumen microbial ecology.

Probiotics improve productivity; increase milk yield, induce better nutrient digestion and enhance growth rate in ruminants.

6.3.4 Health

Apart from their use in improving the performance of ruminant animals, probiotics have been effective in improving animal health. Apas et al. (2010) demonstrated that a probiotic containing *L. reuteri* DDL 19, *L. alimentarius* DDL 48, *E. faecium* DDE 39 and *Bi. bifidum* DDBA (at the ratio of 1:1:1:1), isolated from the faeces of healthy goats, when fed to weaned goats (dose rate of $2x10^9$ cfu/animal/day) reduced the number of pathogenic bacteria (*Salmonella* and *Shigella*) in faeces.

6.3.4.1 Rumen acidosis

The pH of the rumen may drop below the optimum range, following consumption of a diet with a high proportion of non-structural carbohydrates (starch) and/or decreased proportion of fibre (Duffield et al., 2004); short chain fatty acid (SCFA) accumulate and unbalance the buffering capacity of the rumen (Plaizier et al., 2008). The condition is referred to as sub-acute ruminal acidosis (SARA) when the pH decreases below 5.6 and remains between 5.2 and 5.6 for at least 3 hours per day (Gozho et al., 2005). This condition is economically very important as milk production by the suffering animal is reduced due to loss of appetite, diarrhoea, dehydration, debilitation, impaired rumen motility and impaired fibre digestibility (Duffield et al., 2004, Plaizier et al., 2008). Lactic acidosis is the more severe form of ruminal acidosis where the pH drops below 5.2 due to accumulation of lactate (Owens et al., 1998).

Probiotics are effective in preventing or treating ruminal acidosis. Application of *Propionibacterium* P63, *L. plantarum* strain 115 and *L. rhamnosus* strain 32 to the rumen directly via a rumen cannula at the rate of 1×10^{11} cfu/animal/day, a very high dose, was effective in stabilizing rumen pH and preventing acidosis artificially induced by three days of concentrate challenge (wheat, corn or beet pulp) in sheep (Lettat et al., 2012). It was hypothesized that stability in ruminal pH was achieved by the probiotics modulating rumen microbes so that their capacity to hydrolyse cellulose was increased and lactic-acid producing bacteria were inhibited. Similarly, the lactate utilising bacteria, *Megasphaera elsdenii* (Prabhu et al., 2012) was effective in preventing lactic acid accumulation during *in vitro* fermentation (Kung and Hession, 1995). Klieve et al. (2003) demonstrated that the probiotic *M. elsdenii* strain YE34 could be established in the rumen of cattle fed high grain diets, inducing the establishment of lactic acid-utilizing bacteria some 7-10 days earlier than in non-inoculated cattle. Interestingly, ruminants fed high grain diet (barley) have *Ruminococcus bromii* as a dominant bacterial population in the rumen and this bacterium has been suggested as a potential probiotic to enhance the efficiency of starch utilization in grain fed cattle (Klieve et al., 2007). Similarly, yeast *S. cerevisiae* decreased the lactic acid concentration in the rumen of lactating Holstein cows (Marden et al., 2008), which may prevent ruminal acidosis (Thrune et al., 2009). In contrast, Hristov *et al* (2010) found no effect of S. cerevisiae culture, containing metabolites of yeast fermentation, on ruminal fermentation.

Even though probiotics were found effective to prevent rumen acidosis, it has been difficult to establish stable populations of potential probiotics in the rumen. Chiquette *et al.* (2007) tried to establish *Ruminococcus flavefaciens* NJ by adding the bacterium with the probiotic yeast *S. cerevisiae* hoping it would stabilize ruminal conditions to favour the establishment of the inoculated

bacteria. Similarly, *Ruminococcus bromii* YE282 was inoculated with *Megasphaera elsdenii* YE34 as an alternative starch utilizing bacterium in steers (Klieve et al., 2012). There was no effect on acidosis and only *M. elsdenii* YE34 established in the rumen environment. However, Jones and Megaritty (Jones and Megarrity, 1986) successfully introduced and established an exogenous microbe *Synergesties jonesii* (Allison et al., 1992) in the rumen of goat and subsequently cattle (Pratchett et al., 1991, Jones et al., 2009) which at the time was believed to prevent toxicity due to the amino acid mimosine when leaves of the leguminous shrub Leucaena are used as fodder. But another mechanism may be a buildup of tolerance to mimosine and its toxic breakdown product 3,4-dihydroxypyridine and its detoxification in the liver (Halliday et al., 2013).

6.3.4.2 Reduced shedding of E. coli O157:H57

E. coli O157:H57, the Shiga-toxin producing *E. coli*, is an important zoonotic pathogen causing haemorrhagic diarrhoea and haemolitic uremic syndrome (HUS) which can result in acute kidney failure in children (Karmali et al., 2010). Contamination of animal products (meat, milk, egg) from infected animals with this pathogen is a serious public health issue. Wisener et al. (2014) undertook a meta-analysis of the effect of probiotics in reducing the shedding of *E. coli* O157:H57 in beef cattle and found both the long (> 90 days) and short (< 90 days) term applications were effective. The combination of *L. acidophilus* and *P. freudenreichii* was the most effective probiotic treatment while a dose rate of 10^9 cfu/animal/day was more effective than lower dose rates. Earlier studies had also found that a combination of *L. acidophilus* and *P. freudenreichii* significantly reduced faecal shedding of O157 in cattle (Sargeant et al., 2007).

Similarly, Ohya et al. (2000) developed a probiotic containing *S. bovis* LCB6 and *L. gallinarum* LCB 12, isolated from adult cattle, that was effective in eliminating the shedding of O157. They postulated that a significantly increased concentration of SCFA, particularly acetic acid, in the GIT could be the reason for the inhibition of O157.

6.3.4.3 Calf scours

Stress in young calves frequently leads to scours or diarrhoea and weight loss. The stressors are often animal husbandry practices including weaning, vaccination, dehorning, castration, tagging etc. or high temperature. In addition, the rumen and its microbial population are not fully-developed and functional in the early days of life.

Probiotics can reduce such problems in young calves, but results were variable. The effect of the probiotic *L. acidophilus* in reducing the incidence of diarrhoea in young dairy calves was reported

as early as 1977 (Bechman et al., 1977). Other studies using LAB probiotics, also obtained a reduced incidence of diarrhoea in calves (Abe et al., 1995, Abu-Tarboush et al., 1996, Jatkauskas and Vrotniakiene, 2010). Similarly, the incidence of diarrhoea per calf, the duration of each event of diarrhoea and total number of days of diarrhoea in dairy calves from week 4 to 12 raised in sub-tropical summer was significantly reduced by dietary supplementation of *B. amyloliquefaciens* strain H57 (Le et al., 2016). In contrast, Cruywagen et al. (1996) did not find a reduced incidence of diarrhoea, when young dairy calves were fed *L. acidophilus* with milk replacer at the rate of 10^8 cells per animal per day. However, the probiotic did prevent weight loss in the treated calves, while the control calves lost weight. Riddell et al. (2010) also found no effect on the incidence and duration of diarrhoea in young calves from feeding with milk replacer the commercial probiotic (Bioplus 2B) containing *B. licheniformis* (DSM 5749) and *B. subtilis* (DSM 5750). Stress in animals causing dysbiosis or microbial imbalance in the GIT may be needed for the probiotic to benefit calf health.

Probiotics can reduce diseases of ruminants particularly those related to the disturbance of rumen pH (e.g. acidosis), calf scours and pathogenic E. coli. Probiotics are believed to stabilize *ruminal pH by modulating rumen microbes. Lactate utilizing bacteria (e.g.* Megasphaera elsdenii*) could potentially be used to prevent the accumulation of lactic acid in the rumen. However, the establishment of such microorganisms in the rumen is difficult. Similarly, probiotics are effective in reducing the incidence of calf scours by preventing ruminal dysbiosis. Probiotics are also effective in reducing the faecal shedding of the shiga-toxin producing* E. coli *O157:H57. However, these responses to use of probiotics are highly variable and reflect differences in microorganisms (species, strains) used as probiotics and differences in animal husbandry practices (nutrition, housing etc.).*

6.3.5 Rumen fermentation

The meta-analysis of the application of yeast probiotics (containing at least one strain of *S. cerevisiae*) in ruminants by Desnoyers *et al.* (2009) demonstrated that live yeast significantly increased rumen concentrations of SCFA and increased rumen pH, but the results were highly variable. Although yeast supplementation moderately decreased rumen lactic acid concentration, there was no effect on the acetate to propionate ratio. However, the effect of yeast supplementation on rumen fermentation varied with the proportion of concentrate in the diet. In general, "The positive effect of yeast supplementation on rumen pH increased with the percentage of concentrate in the diet and with the Dry Matter Intake (DMI) level" (Desnoyers et al., 2009). Similarly, yeast probiotics increased the concentration of SCFA with increased CP concentration and DMI

(Desnoyers et al., 2009). The higher the proportion of concentrate and neutral detergent fibre in the diet, the better the digestibility of organic matter resulting from the live yeast supplementation (Desnoyers et al., 2009).

It has been postulated that yeast based probiotics in ruminants increase the number of cellulolytic bacteria which affects the microbial fermentation resulting in higher cellulose degradation and increased microbial protein production (Dawson et al., 1990, Newbold, 1996, Chaucheyras-Durand et al., 2008).

Using quantitative real-time PCR, Ding *et al.* (2014) demonstrated that *S. cerevisiae* increased the total number of rumen bacteria in crossbred steers fed alfalfa mixed with concentrates, but the number of rumen fungi and protozoa did not change. The percentage of *Selenomonas ruminantium*, a lactate utilizing bacterium, increased while the percentage of *Ruminobacter amylophilus*, a starch-degrading bacterium, decreased.

Saccharomyces cerevisiae *based probiotics affect rumen fermentation resulting in an increased concentration of SCFA and increase in rumen pH with moderate reduction in rumen lactic acid concentration. These probiotics generally increase the population of cellulolytic bacteria which may result higher cellulose degradation. Generally, the greater the proportion of concentrates in the diet, the more beneficial the administration of yeast to ruminants. With the intensification of the animal production system, use of concentrate has increased. In this context, probiotics may increasingly become a component in intensive ruminant production systems.*

6.3.6 Probiotics with roughage based diet

Most ruminant animal production occurs on low quality roughage and the improvement of digestibility with the use of probiotics is of much interest, even though at present it is only animals fed high quality diets where probiotics could be readily applied.

Yeast probiotics can increase the population of cellulolytic bacteria in the rumen(Dawson et al., 1990, Harrison et al., 1988) which may result in an increased rate of fibre digestion and increased microbial protein turnover, hopefully improving animal performance(Newbold, 1996). However, increase in cellulolytic bacteria may not always result in increased fibre digestion as their activity depends on rumen pH (Russell and Wilson, 1996). Dawson *et al.* (1990) found an increase in the population of cellulolytic bacteria in the rumen of Jersey steers, when a high roughage based diet was supplemented with either *S. cerevisiae* or a combination of *S. cerevisiae*, *L. acidophilus*, and *E. faecium.*

The effects of yeast on rumen fermentation in animals with roughage-based feed are variable. Dietary inclusion of *S. cerevisiae* and/or *Armillaria heimii* (white rot fungi) in sheep increased the dry matter intake, metabolisable energy intake and digestibility of neutral detergent fibre(Mpofu and Ndlovu, 1994). Potentially digestible neutral detergent fibre, crude protein and dry matter of alfalfa hay, cornstalk and coffee hull fed to fistulated Holstein steers was increased with the supplementation of *S. cerevisiae* (Roa et al., 1997). In contrast, addition of yeast to cattle fed a high fibre (barley straw based) diet (Moloney and Drennan, 1994) or high grain diet (Mir and Mir, 1994) did not affect the digestibility of dry matter and neutral detergent fibre and decreased the digestibility of crude protein. Supplementing a sugar cane tops based diet for sheep with yeast did not improve rumen fermentation and digestibility although rumen pH decreased (Arcos-García et al., 2000).

Although probiotics especially Saccharomyces cerevisiae *can improve digestibility of low quality roughage by ruminants, the results are inconsistent. Further study with a wider selection of well characterised probiotic microorganisms (including bacteria) and animal feed constituents, including non-conventional feed resources like agricultural by-products, is needed to assess the benefits.*

7 Safety of probiotics and potential public health risks

The safety of probiotics is discussed in general terms and is not specific to those used in animal feed. The possibility of probiotics used in animal feed entering the human food chain cannot be ruled out. However, there is very little information available about the risk of human food “contamination” with probiotics used in animals.

The microbial genera and species used as probiotics in animal feed are generally considered safe. The most serious risk posed by probiotic microbes in feed are i) transfer of antibiotic resistance due to the presence of transmissible antibiotic resistance genes/determinants in some probiotic bacteria and ii) infections from the probiotic microorganisms and presence of enterotoxins and emetic toxins in probiotic bacteria.

Most publications relating to probiotics deal with their efficacy rather than safety. Most of the information about the safety of probiotics is based on *Lactobacillus* and *Bifidobacterium* (Shanahan, 2012, Hempel et al., 2011). Therefore more research is required in relation to the safety of probiotics.

Shanahan (2012) highlights the limitations of claims made about the safety of probiotics in general and especially the safety of any particular probiotic. According to Shanahan (2012):

1. Safety assessment and information on a particular probiotic strain cannot be generalized to similar probiotics (even with a different strain of the same species), as each probiotic requires safety and risk assessment on a case-by-case basis.

2. The adverse effects and the severity of the effects of a probiotic could be context specific and depend on the susceptibility (immunity) and physiological state of the host (animals or human). Therefore, probiotic strains deemed to be safe in certain conditions may not be safe in other conditions. For example, the prematurely born and immunologically compromised host could be at greater risk than the host born at term.

3. No probiotic can be regarded as 100% safe or with zero risk as is the case with drugs.

4. Public awareness about the risk from probiotics is limited and there is a need for proper risk benefit analysis and communication of this to the user/consumer of the probiotics.

5. The contamination of probiotics with unwanted microbes or substances is an important safety and quality issue as with the safety and quality of probiotics microorganisms *per se*. Sometimes, hazards associated with contaminants may be a more important issue than the specific quality of the probiotics.

In 2010, the Agency for Healthcare Research and Quality under the U.S. Department of Health and Human Services conducted a systematic study of published data and information on the safety of probiotics. The study concluded that “there is a lack of assessment and systematic reporting of adverse events in probiotic intervention studies, and interventions are poorly documented” (Hempel et al., 2011). Although there are many publications on the safety of probiotics, the evidence available is not enough to address all the safety issues and precludes a declaration of probiotics as universally safe or unsafe (Hempel et al., 2011).

Although microorganisms used as probiotics in animal feed are generally safe some of the bacterial species and/or strains pose risks mainly by transmission of antibiotic resistance to pathogenic microbes or production of enterotoxins (Anadón et al., 2006).

7.1 Risk associated with probiotics

Although microorganisms used as probiotics in animal feed are relatively safe, precaution should be taken to protect animals, humans and the environment from potentially unsafe microorganisms.

Theoretically, risks associated with the use of probiotics in animal feed are as follows (Marteau, 2001, Doron and Snydman, 2015, FAO and WHO, 2002):

1. Infection (gastrointestinal or systemic) of the animal fed the probiotic
2. Infection (gastrointestinal or systemic) of the consumers of animal products produced by animals fed probiotics
3. Transfer of antibiotic resistance from probiotics to other pathogenic microorganisms
4. Release of infectious microorganisms or noxious compounds to the environment from the animal production system
5. Infection (gastrointestinal or systemic) of the handlers of animal or animal feed
6. Skin and/or eye and/or mucus membrane sensitisation in the handlers of probiotics
7. Detrimental metabolic or toxic effects in the host due to the production of toxins by the microorganisms contained in probiotics
8. Hyper-stimulation of the immune system in susceptible hosts

7.1.1 Assessment of risk

The microorganisms considered for use as probiotics in animal diets should be assessed against the above-mentioned risks. The microorganism under consideration need to be identified to strain level (Figure S1). The particular strain of microorganism should not have been associated with any infection in humans or animals. Similarly, the putative probiotic should not harbour transferable antibiotic resistance genes. Microorganisms which either produce toxins or cause hyper-stimulation of the immune system in the host are generally not suitable for probiotics.

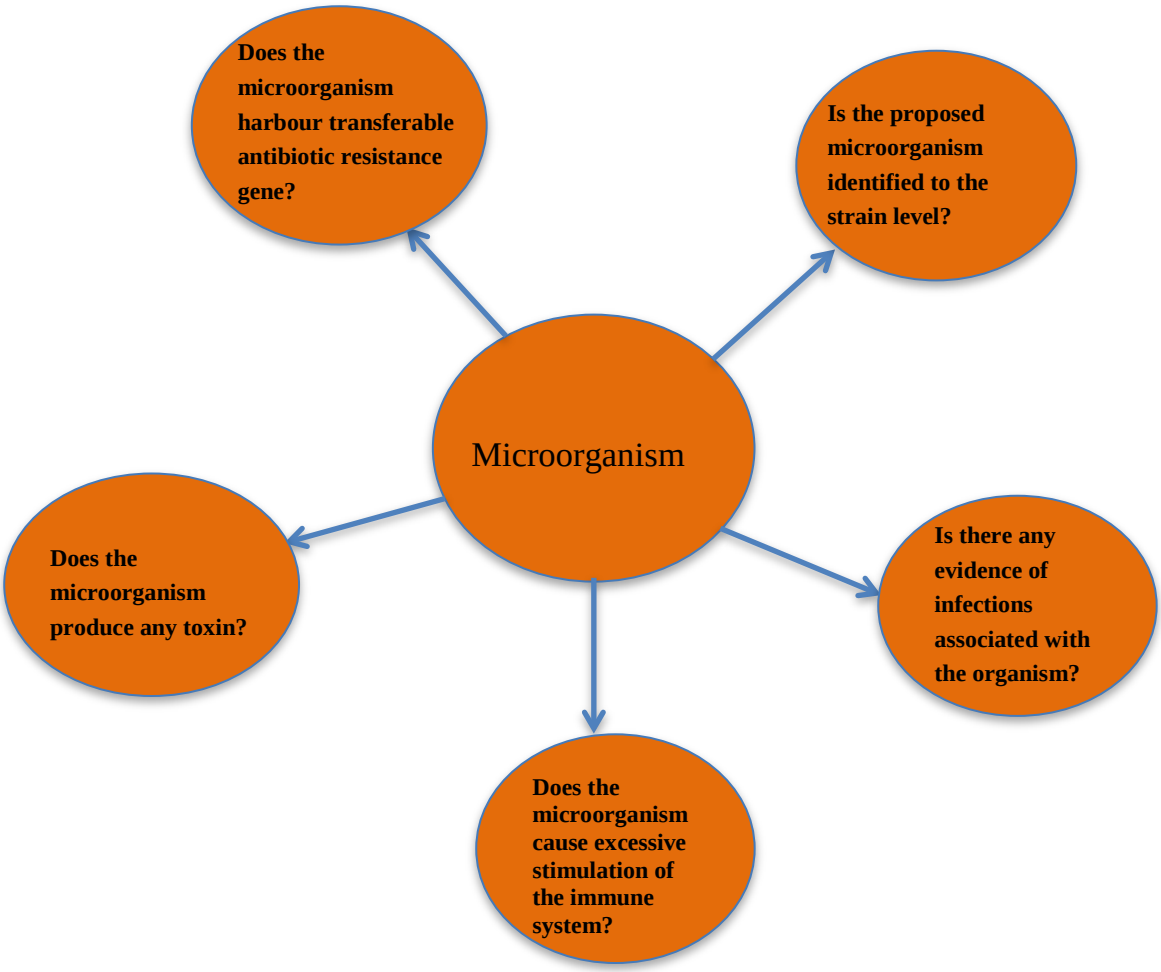

Figure S1: Major questions to be addressed when assessing the safety of microorganisms being considered for use as probiotics in animal feed

7.1.1.1 Qualified Presumption of Safety (QPS): European approach for the assessment of the safety of probiotics

In 2002, a group of scientists, consisting of members of the former Scientific Committees on Animal Nutrition, Food and Plants of the European Commission, developed the concept of QPS to address a need for a tool which selectively prioritizes the assessment of risk of the use of a particular microorganism in food and feed (European Food Safety Authority, 2007). EFSA has been using this concept since 2007 as a generic risk assessment tool to assess the safety of a microorganism intended to deliberately enter the food chain. According to this concept, if microorganisms of certain predetermined taxonomic groups either do not pose any safety risk or risk can be clearly defined and eliminated, the group can be designated as a group with QPS status. Any particular microorganism intended to be introduced into the food chain, which can be unequivocally identified and have QPS status, may not be the subject of a detailed pre-market

safety assessment other than satisfying predetermined specific qualifications (European Food Safety Authority, 2007). Thus, resources (time and money) could be prioritized to those microorganisms, which do not fulfil the above mentioned qualifications and have an uncertain risk status, thus avoiding the need to investigate microorganisms with proven safety. Microorganisms, not listed as having QPS status, would undergo a detailed pre-market safety assessment. QPS status is only given to microorganisms but not to any product containing such microorganism (European Food Safety Authority, 2007). QPS status is maintained up to the species level.

Safety assessment of a particular microorganism or a taxonomic group to decide QPS status is usually done on the basis of four pillars of QPS assessment (European Food Safety Authority, 2007) as outlined in Figure S2. EFSA has listed more than 100 species of microorganisms under QPS status; which are broadly categorized into i) Gram-positive non-sporulating bacteria, ii) *Bacillus* species and iii) yeasts.

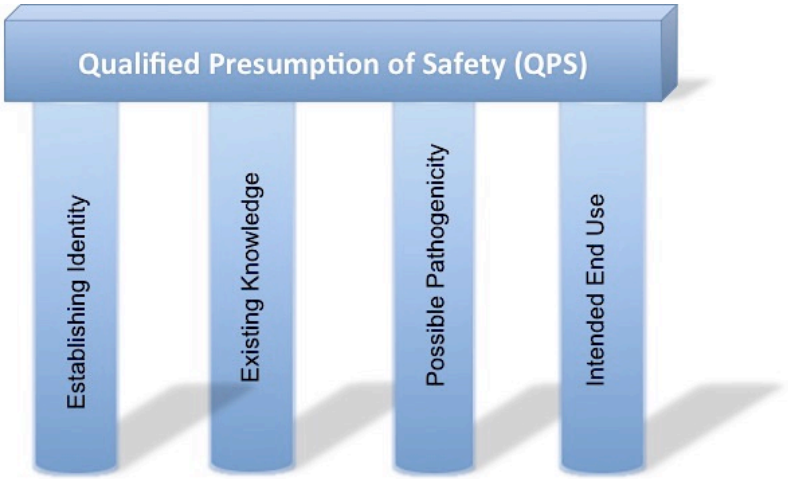

Figure S2: Assessment of a microorganism or a taxonomic unit to assign QPS status

Probiotic use is not without risk. Probiotics could be responsible for a range of hazards in animal health, human health and the environment ranging from mild reactions to serious life threatening infections. Moreover, information about safety of one particular microorganism should not be applied to other closely related microorganisms. Present levels of information are not sufficient to declare any group of probiotics 100% safe. Therefore, risk assessment on a case by case basis is recommended.

7.2 Safety of microbial genera commonly used as probiotics

7.2.1 Lactobacillus and *Bifidobacterium*

Lactobacillus and *Bifidobacterium* are probably the safest microorganisms used as probiotics because i) these microorganisms have been safely used traditionally in various fermented food (Shortt, 1999); ii) these microorganisms are naturally present in the GIT and other sites in humans (Human Microbiome Project Consortium, 2012, Huse et al., 2012) and animals (Yeoman and White, 2014, Yeoman et al., 2012) in large quantities; and iii) infections associated with these microorganisms are extremely rare (Gasser, 1994, Saxelin et al., 1996). *L. acidophilus* and *L. bulgaricus* have been categorized as "Generally Regarded as Safe" by US Food and Drug Administration (FDA) (USFDA, 2013). Nevertheless, LAB have been reported to cross the intestinal mucosal barrier resulting in bacteraemia and inflammation of the heart muscle (endocarditis) in susceptible people with compromised immunity (Soleman et al., 2003, Cannon et al., 2005, De Groote et al., 2005, LeDoux et al., 2006). However, the chance of this happening is extremely rare and reported to be less than 1 per 10^6 (Sanders et al., 2010). These rare incidences of lactobacillaemia can be very serious or even fatal (Saxelin et al., 1996, Husni et al., 1997).

In a small number of cases, incidences of endocarditis and other internal infections characterized by internal inflammatory lesions (e.g. liver abscess) were reported to be associated with the consumption of large quantities of dairy products containing *L. rhamnosus* GG as a probiotic (Cannon et al., 2005, Rautio et al., 1999). However, the nature of risk from the probiotics used in animal diets and those from human food could be entirely different.

It is often difficult to define the clinical significance of the occurrence of Lactobacillus in clinical specimens as mostly the infection is opportunistic due to compromised immunity of the host (European Food Safety Authority, 2007). Therefore, safety assessment tools may not be able to exclude these types of opportunistic infections (European Food Safety Authority, 2007). There are 35 species of Lactobacillus included in the Qualified Presumption of Safety (QPS) list of the European Food Safety Authority (EFSA) (EFSA BIOHAZ Panel, 2013). *L. plantarum* KKP/593/p and *L. rhamnosus* KKP 825 were the latest addition to be authorised as safe to use as feed additive for chickens (EFSA FEEDAP Panel, 2016).

In the context of *Lactobacillus* taxonomy being updated with advances in knowledge from molecular biology, some of the previous claims about *Lactobacillus* and its aetiology in clinical disease may have been wrongly reported due to misidentification of the causative agent as

Lactobacillus (Salminen et al., 2002, Bernardeau et al., 2008).

Like *Lactobacillus*, *Bifidobacterium* are also another safe choice as probiotic bacteria. They are very rarely associated with infections in healthy hosts. *Bifidobacterium adolescentis, Bi. animalis Bi. bifidum, Bifidobacterium breve* and *Bi. longum* have been given Qualified Presumption of Safety (QPS) status by the European Food Safety Authority (EFSA) (EFSA BIOHAZ Panel, 2013). However, incidences of bifidobacteria being found associated with infections have been reported in immunocompromised hosts (Jenke et al., 2011, Barberis et al., 2012, Ohishi et al., 2010).

Lactobacillus and Bifidobacterium species are generally considered the safest choice as probiotics. Nevertheless, some very rare cases of infections (e.g. endocarditis, lactobacillaemia) have been reported in immunocompromised people.

7.2.2 *Bacillus*

Spore forming bacteria, particularly different species from the genus *Bacillus,* are becoming increasingly popular as probiotics for use in animal feed, due to their robustness in withstanding high temperatures making them easier to handle during manufacture, storage, and transportation of feed. The European Commission has identified 13 *Bacillus* species and given QPS status including *B. subtilis, B. amyloliquefaciens, B. licheniformis, B. coagulans* and *B. megaterium* which are used in probiotics for animal feed (EFSA BIOHAZ Panel, 2013). These *Bacillus* species were identified as safe mainly due to an absence of enterotoxins and emetic toxins (EFSA BIOHAZ Panel, 2013).

The use of spore forming bacteria as probiotics is not risk-free as some *Bacillus* species (e.g. *B. anthracis, B. cereus, B. thuringiensis* etc.) are pathogenic in humans and animals (Little and Ivins, 1999, Kotiranta et al., 2000, Hernandez et al., 1998, Damgaard et al., 1997, Raymond et al., 2010). Although there is detailed information about the pathogenicity of *B. anthracis* and *B. cereus,* there is no evidence for pathogenic effects for other endospore forming bacteria.

B. cereus produces the emetic toxin cereulide and enterotoxins haemolysin BL (Hbl) and non-haemolytic enterotoxin (Nhe) and cytotoxin K (CytK), which cause serious illness in humans (Schoeni and Lee Wong, 2005, Granum and Lund, 1997). From et al. (2005) screened 333 strains from different species of *Bacillus* to investigate the production of enterotoxins and emetic toxins. Eight *Bacillus* strains belonging to *B. subtilis, Bacillus mojavensis*, *Bacillus pumilus,* and *Bacillus fusiformis* were found to produce cytoxic and emetic toxins. In addition, some *Bacillus* species such as *B. cereus* has been reported to cause mastitis in cattle (Parkinson et al., 1999) and *B. licheniformis* was associated with abortion in cattle (Agerholm et al., 1997).

Some Bacillus species used as probiotics (e.g. Bacillus subtilis*) produce cytotoxic and emetic toxins. Therefore, detailed safety studies are recommended for these microbial strains before use as probiotics.*

7.2.3 Enterococcus

In spite of several examples of beneficial effects of *Enterococcus* probiotics in animals and humans and a long history of safe use, these bacteria have been associated with several infections in humans and the presence of transferable antibiotic resistance determinants (Franz et al., 2011, Franz et al., 2003, Franz et al., 1999). *Enterococcus* species, particularly *E. faecalis* and *E. faecium*, are associated with community and hospital acquired infections and were amongst the most prevalent causes of hospital acquired infections in the 1990s (Spera and Farber, 1992). Several virulence factors from *Enterococccccus* have been identified and are associated with either colonisation, invasion or production of pathological lesions (Franz et al., 2011). These bacteria are also opportunistically associated with urinary tract infection, endocarditis and enterococcal bacteraemia in humans (Morrison et al., 1997).

There are many commercial probiotic products available on the market, which contain *Enterococcus* bacteria (Abdel-Rahman et al., 2013, Mountzouris et al., 2010, Wideman Jr et al., 2012, Landy and Kavyani, 2013, Khaksar et al., 2012). Due to the widespread prevalence of enterococcal infections and proven virulence of the bacteria, the EU has not given this genus QPS status, thus requiring safety assessment on a case by case basis (EFSA BIOHAZ Panel, 2013).

Enterococcus bacteria are associated with community and hospital acquired infections and therefore stringent safety evaluations are required before use as probiotics.

7.3 Antibiotic resistance associated with probiotics

The emergence of multi-drug resistant pathogens is now one of the greatest threats to public health around the world (Sengupta et al., 2013). Although the initial emergence of antibiotic resistance is believed to be the outcome of evolution, imprudent use of antibiotics is believed to be the major cause of widespread antibiotic resistance (Laxminarayan et al., 2013, Davies and Davies, 2010). Antibiotic resistance genes are generally present in plasmids, transposons and integrons of bacteria and can transfer from one bacterium to another (intra- or inter-species) by mechanisms of horizontal gene transfer (Alekshun and Levy, 2007, van Reenen and Dicks, 2011, Santagati et al., 2012, Blair et al., 2015). Transposons are the most important mobile element in the bacterial cell and responsible for inter-species transfer of antibiotic resistance genes (Wozniak and Waldor, 2010).

The nature of antibiotic resistance determinants is more important than antibiotic resistance *per se* because all of the determinants of antibiotic resistance may not be transferrable (Davies and Davies, 2010).

Although resistance to antifungal drug in pathogenic fungi is becoming a problem of increasing importance (Pfaller and Diekema, 2004, Morschhäuser, 2010), the mechanism of transfer of resistance determinants in fungi is different from antibiotic resistance in bacteria (Anderson, 2005). In fungi, horizontal transfer of drug-resistance gene (and other genes) does not take place easily particularly among divergent taxa (Anderson, 2005). Therefore, there is no evidence about the risk of transfer of antifungal resistance from yeast probiotics.

The GIT of animals contains a complex microbial ecosystem with diverse and large numbers of microorganisms. Proximity of bacteria to each other in complex microbial ecosystem like intestine can favour the transfer of genetic material including antibiotic resistance gene from non-pathogenic to pathogenic microorganisms (Aarts and Margolles, 2015). The possibility of the transfer of antibiotic resistance genes to potential enteric pathogens in the GIT cannot be excluded (Aarts and Margolles, 2015). Therefore, if a bacterium intended to be used as an animal probiotic is harbouring transferable antibiotic resistance genes this could be a medium for transfer of antibiotic resistance to the environment and humans (González-Zorn and Escudero, 2012).

7.3.1 Antibiotic resistance in *Lactobacillus*

Although *Lactobacillus* are considered one of the safest bacteria used as probiotics, many species of these bacteria harbour one or more antibiotic resistance genes (Mathur and Singh, 2005, Ammor et al., 2007, Gueimonde et al., 2013). The possibility of horizontal transfer of these antibiotic resistance genes and their association with mobile elements (plasmids, transposons and integrons) has not been extensively studied. Nevertheless, some of the food borne species of *Lactobacillus* have antibiotic resistance genes which are capable of being transferred horizontally to pathogenic bacteria and are associated with mobile elements (Table S3) (Tannock et al., 1994). Some *Lactobacillus* species have acquired antibiotic resistance genes from other Gram positive bacteria (Shrago et al., 1986, Tannock, 1987).

The *Lactobacillus* species reported to harbour transferable antibiotic resistance genes, are components of some commercial probiotics (Mookiah et al., 2014, Bai et al., 2013, Daskiran et al., 2012, Biloni et al., 2013, Mountzouris et al., 2010). However, the presence of such elements in those particular probiotic strains has not been established. Tetracycline resistance genes (*tet*) are the

most frequent in *Lactobacillus* (Ammor et al., 2008c) while aminoglycoside resistance genes and β-lactam resistance genes (*blaZ*) are least frequent (Aquilanti et al., 2007).

Table S6: *Lactobacillus* species with antibiotic resistance genes capable of horizontal transfer

Species	Source	Antibiotic resistance gene(s)	Associated mobile elements	References
L. brevis	Dairy	*tet*(M)	Not known	(Nawaz et al., 2011)
L. fermentum	Dairy	*erm*(B), *msr*C, *erm*(C), *erm*(T), *tet*(K), *tet*(L)	Plasmid, transposon	(Nawaz et al., 2011, Thumu and Halami, 2012, Gfeller et al., 2003)
L. paracasei	Dairy	*tet*(M)	Tn*916*	(Devirgiliis et al., 2009)
L. plantarum	Dairy, Vegetables	*tet*(M), *erm*(B), *tet*(W), *tet*(L)	Plasmid	(Nawaz et al., 2011) (Feld et al., 2009, Thumu and Halami, 2012)
L. salivarius	Fermented food, Vegetable	*erm*(B), *tet*(W), *tet*(M), *tet*(O), tet(L)	Not known	(Nawaz et al., 2011, Thumu and Halami, 2012)
L. reuteri	Fermented food, Poultry	*erm*(B), Cat-TC, *tet*(W)	Plasmid	(Lin et al., 1996, Thumu and Halami, 2012)

7.3.2 Antibiotic resistance in *Bifidobacterium*

Some species of *Bifidobacterium* demonstrate phenotypic antibiotic resistance characters and have associated antibiotic resistance genes (Ammor et al., 2008b) but most are not associated with mobile elements and thus are non-transferable. These bacteria are therefore suitable for use in the food chain as probiotics in animal feed (Ammor et al., 2008a, Van Hoek et al., 2008, Kazimierczak et al., 2006, Flórez et al., 2006). However, several species and strains of *Bifidobacterium* including *B. longum* and *B. animalis subsp. lactis* harbours the antibiotic resistance gene *tet(W)*, which is capable of intra-species transfer among *Bifidobacterium* (Gueimonde et al., 2013, Aarts and Margolles, 2015).

7.3.3 Antibiotic resistance in *Bacillus*

Antibiotic resistance has frequently been reported in *Bacillus. B. subtilis,* a frequently used probiotic can harbour conjugative transposons (e.g. Tn5397), which can transfer resistance to tetracycline encoded by the *tet(M)* gene (Mullany et al., 1990, Roberts et al., 1999). Phelan et al. (2011) reported another transferable tetracycline resistance gene *tet(L)* in a *Bacillus sp.* encoded by a plasmid. *B. subtilis* can contain the macrolide-lincosamide-streptogramin B (MLS) resistance determinants on a plasmid (Monod et al., 1986). Macrolides are a very important class of antibiotics widely used to control human and animal infections. The MLS determinant is homologous to the *erm(C)* gene, one of 19 analogous *erm* resistance genes (Monod et al., 1986). The most prevalent antibiotic resistance gene is *erm(D)* which encodes the determinants for the resistance to MLS (Gryczan et al., 1984, European Food Safety Authority, 2007). However, transferability of the determinants encoded by this gene has not been confirmed (European Food Safety Authority, 2007).

Transfer of antibiotic resistance genes to potential pathogenic microorganisms is one of the serious risks associated with probiotics, as many bacterial species used as probiotics harbour transferable antibiotic resistance genes. Therefore, stringent quality assurance measures are recommended in this regard using microbes as probiotics only with proven absence of transferable antibiotic resistance genes. Lactobacillus, Bacillus and Enterococcus have greater risk as many species of these genera have transferable antibiotic resistance genes while Bifidobacteria carry less risk as most of the resistance genes in these bacteria are non-transferable. However, the status of antibiotic resistance genes in microbial strains used as probiotics has not been determined. Presence of antibiotic resistance genes may not be a serious issue if such genes are intrinsic in chromosomes and not transferable. Nevertheless, precautions should be taken to avoid microbes with acquired genes to use as probiotics.

8 Labelling of probiotics used in animal feed

Labels in the packaging of commercial probiotic products should provide information about content, positive effects of the products, date of expiry, dose rates, contraindications (if any) etc. However, commercial probiotics are often inadequately or incorrectly labelled. Weese (2003) suggested that an ideal probiotic label “should state the organisms that are present to the strain level, correctly spell and identify the contents, state the number of live organisms, and guarantee that the stated number would be present at the time of expiry”. Another piece of essential

information that should be present on the label is the dose rate to be used for different categories of animals. This was often neglected on the labels (Weese, 2003).

Few studies have examined the quality and authenticity of probiotic labelling. Weese and Martin (2011) found that the labelling of commercial probiotics was very poor. The common errors in the labelling were failing to mention specific names of microorganisms in the product, failing to give number of viable microorganisms in the product, giving conflicting information, not mentioning expiry date, and misspelling the microbial name (Weese and Martin, 2011, Weese, 2003).

On labels of commercial probiotics produced for humans and animals, and marketed through health food stores, pharmacies, grocery stores, companion pet stores and veterinary clinics, some manufacturers use vaguely descriptive terms like “dried lactobacillus,” “lactobacillus cultures”, “probiotic cultures”, “fermentation products” etc. instead of specific names of the microorganisms in the product (Weese, 2003). Although a significant proportion of the commercial products included the name of the microorganism(s) on the label, only eight out of twenty five (32%) products studied in Canada had a label with the correct names of microorganisms and the number of viable organisms in the product (Weese and Martin, 2011). A significant number of producers misspelled the name of microbes, including using the obsolete name and even listing the names of microbes which did not exist (Weese, 2003). Very few of the products were reported to have labels with the name of microorganisms to the strain level (Weese, 2003).

Similarly, not all products had information about the number of viable microorganisms and even if the information was present it was not clear whether the specified quantity was at the time of manufacture or at the time of expiry (Weese, 2003). More serious was the problem that only four out of 15 (27%) products which mentioned the quantity of viable microorganisms, actually met their claimed quantity. Ironically, there was even a product with no viable microorganisms at all, despite claiming to have 14 million cfu/capsule. Only a small proportion (8%) of the studied products had both a satisfactory label and the quantity of viable bacteria as claimed on the label (Weese and Martin, 2011).

The most serious probiotic labelling errors occurred from wrong information, such as labelling the product as yeast instead of *Lactobacillus sp*., or claiming to have bacteria present that were not detected in the product, or claiming to have more bacteria than were actually present in the products (Lata et al., 2006, Weese, 2002). Inclusion of microorganisms with no proven probiotic effects and inclusion of potentially pathogenic microorganisms in commercial products were other serious issues noted (Weese, 2002).

The objectives of probiotic labelling should be to provide the users with all necessary information to properly handle, store, transport and use the products with necessary precautions to minimize hazards associated with the product. The label should be in a language understandable to the intended users. Probiotics with labels only in the English language are commonly marketed in developing countries, where the users may not understand English. Therefore, labels should be tailored to the intended audiences. The label should also assist in making an informed choice by end users.

9 Global regulatory status of probiotics in animal feed

The advancement in the knowledge of the GIT microbial ecology and the mechanism(s) of probiotic action increases the possibility of the introduction of new probiotics. There is therefore, increasing interest in the regulation of these products to protect human health, animal health and the environment. It is also important that the claims made by the manufacturers of probiotics are correct and consumers are appropriately protected.

Unlike other feed additives, probiotics have certain distinctive attributes. Probiotics are living organisms, can be inactivated in the GIT, and may interact with the genetics of the host animals. These factors require probiotics to be regulated more stringently than other feed additives (Hoffmann et al., 2013). Moreover, there is a fine line between whether a probiotic is treated as a feed additive or a therapeutic agent. This affects the way in which the probiotic is regulated.

There are no studies on the release of probiotics into the environment either from animal manures or from other sources in their production and use.

9.1 Codex Alimentarius Commission

Codex Alimentarius Commission (CAC), originally established by FAO and WHO to develop food safety guidelines, has defined a feed additive in “code of practice on good animal feeding - CAC/RCP 54-2004” as “any intentionally added ingredient not normally consumed as feed by itself, whether or not it has nutritional value, which affects the characteristics of feed or animal products” (Codex Alimentarius Commission, 2004), which includes microorganisms, enzymes, acidity regulators, trace elements and vitamins. Therefore, “code of practice on good animal feeding” is the relevant code of CAC to follow as guidelines for the production, processing, storage, transport and distribution of probiotics by member states, in addition to their national legislation, to regulate probiotics.

9.2 United States Food and Drug Administration

The Food and Drug Administration (FDA) is the primary authority within the US Department of Health and Human Services which has a mandate to regulate and oversee the use of foods, medicines (both prescription and over the counter drugs), vaccines, veterinary products, dietary supplements etc. All products under the jurisdiction of the FDA are regulated by one of its six centres based on the category of the products according to the intended use, generally as stated by the manufacturers. Hence, intended use is more important than the contents of the products in determining the nature of regulation of the products.

All livestock feeds, pet foods, veterinary drugs and devices and veterinary biologicals are regulated by the Center for Veterinary Medicine (CVM) under the FDA. The CVM regulates the safety, effectiveness, labelling and distribution of the products under its jurisdiction. In case of any ambiguity or when there is confusion about which product should be regulated by which centre, the Office of Combination Products (OCP) under the FDA provides guidelines. Similarly the Federal Trade Commission regulates the advertising and marketing of the products and may have a role in certain aspects of probiotic regulation.

The FDA uses the phrase Direct-fed Microbial (DFM) products for probiotics used in animal feed. The FDA guidance document (CPG Sec. 689.100) has defined DFM as “products that are purported to contain live (viable) microorganisms (bacteria and/or yeast)” (USFDA, 2015). This FDA guideline has approved the microorganisms listed in the official publication of the Association of American Feed Control Officials (AAFCO) that can be used in DFM (Table S4). Products marketed solely as silage additives are not regulated as DFM. For regulatory purposes, DFM are considered either as fermentation products or yeast products.

The regulation of probiotics by FDA is chiefly determined by the intended use or claim of the product (Table S5). It could be either food/feed or drug or both and regulated accordingly. The probiotics with the following claims are categorized as “new animal drug” and regulated as a drug and need an approved new animal drug application (USFDA, 2015).

- Cure, mitigation, treatment or prevention of diseases
- Affect the structure or function of the body

The approved microorganisms listed in the publication of the AAFCO when marketed as DFM without any therapeutic or structure/function claims are categorized as food and regulated accordingly. The products categorized as food are monitored by the respective State Government rather than FDA unless these products have any safety issue (USFDA, 2015). However, if the

marketed microorganisms are not listed by AAFCO and have no therapeutic or structure/function claims, the product is categorized as a food additive and regulated accordingly.

Table S7: Microorganisms in the official list of AAFCO that are suitable for use in animal feed (Pendleton, 1998)

Aspergillus niger	*Lactobacillus curvatus*
Aspergillus oryzae	*Lactobacillus delbruekii*
Bacillus coagulans	*Lactobacillus farciminis* (swine only)
Bacillus lentus	*Lactobacillus fermentum*
Bacillus licheniformis	*Lactobacillus helveticus*
Bacillus pumilus	*Lactobacillus lactis*
Bacillus subtilis	*Lactobacillus plantarum*
Bacteroides amylophilus	*Lactobacillus reuterii*
Bacteroides capillosus	*Leuconostoc mesenteroides*
Bacteroides ruminocola	*Pediococcus acidilacticii*
Bacteroides suis	*Pediococcus cerevisiae (damnosus)*
Bifidobacterium adolescentis	*Pediococcus pentosaceus*
Bifidobacterium animalis	*Propionibacterium acidpropionici* (cattle only)
Bifidobacterium bifidum	*Propionibacterium freudenreichii*
Bifidobacterium infantis	*Propionibacterium shermanii*
Bifidobacterium longum	*Saccharomyces cerevisiae*
Bifidobacterium thermophilum	*Enterococcus cremoris*
Lactobacillus acidophilus	*Enterococcus diacetylactis*
Lactobacillus brevis	*Enterococcus faecium*
Lactobacillus buchneri (cattle only)	*Enterococcus intermedius*
Lactobacillus bulgaricus	*Enterococcus lactis*
Lactobacillus casei	*Enterococcus thermophilus*
Lactobacillus cellobiosus	*Yeast*

Table S8: Regulation of Direct Fed Microorganisms (Probiotics) by FDA

Product	Intended use/Claim	Legal status	Regulated as	Regulated by
DFM	Cure, mitigate, treatment or prevention of disease	New animal drug	Drug	FDA
	Affect the structure and function of the body	New animal drug	Drug	FDA
	Without any therapeutic or structure/function claim (microorganisms listed in AAFCO official publication)	Food	Food	State government
	Without any therapeutic or structure/function claim (microorganisms not listed in AAFCO official publication)	Food additives	Food additives	FDA

9.2.1 Generally regarded as safe (GRAS)

The US Food and Drug Administration's Center for Veterinary Medicine (CVM) has a Generally Recognized as Safe (GRAS) notification programme for ingredients in animal feed. According to this programme "any substance that is intentionally added to food" is exempt from regulation as a food additive, if the substance is GRAS. A food additive could get GRAS status either through scientific justification or based on a long history of safe use of the product in animal feed (before 1958).

9.3 European Food Safety Authority (EFSA)

The EU follows a very strict regulation for the assessment of probiotics for which manufacturers of the probiotics should provide evidence of the identity, safety and efficacy of the product which is assessed by a scientific committee of experts (Europen Commission, 2003). Probiotic products can only be marketed following assessment and approval from the scientific committee and authorization under EU regulation (EC) No. 1831/2003, additives for use in animal nutrition. The manufacturers should follow use and labelling conditions to market the product as authorized by the European Commission.

Regulation (EC) No. 1831/2003 of the European Parliament and the Council of 22 September 2003 on additives for use in animal nutrition has classified feed additives into 5 categories: (a) technological additives (b) sensory additives (c) nutritional additives (d) zootechnical additives and (e) coccidiostats and histomonastats (Europen Commission, 2003). Although the word 'probiotics' is not used in the regulation, "microorganisms or other chemically defined substances, which when fed to animals, have a positive effect on the gut flora" are categorized as "gut-flora stabilizers", a functional group under zootechnical additives. Therefore, probiotics in animal feed are regulated as zootechnical additives in the EU. The regulation 1831/2003, legislates the authorization, use, monitoring, labelling and packaging of feed additives.

In April 2008, the EU published commission (EC) No. 429/2008, gave detailed rules for the implementation of Regulation (EC) No. 1831/2003, which details procedures for authorization of new probiotics entering the EU (Europen Commission, 2008) as outlined in Figure 3. Authorization granted according to this legislation is valid for 10 years and should be renewed thereafter.

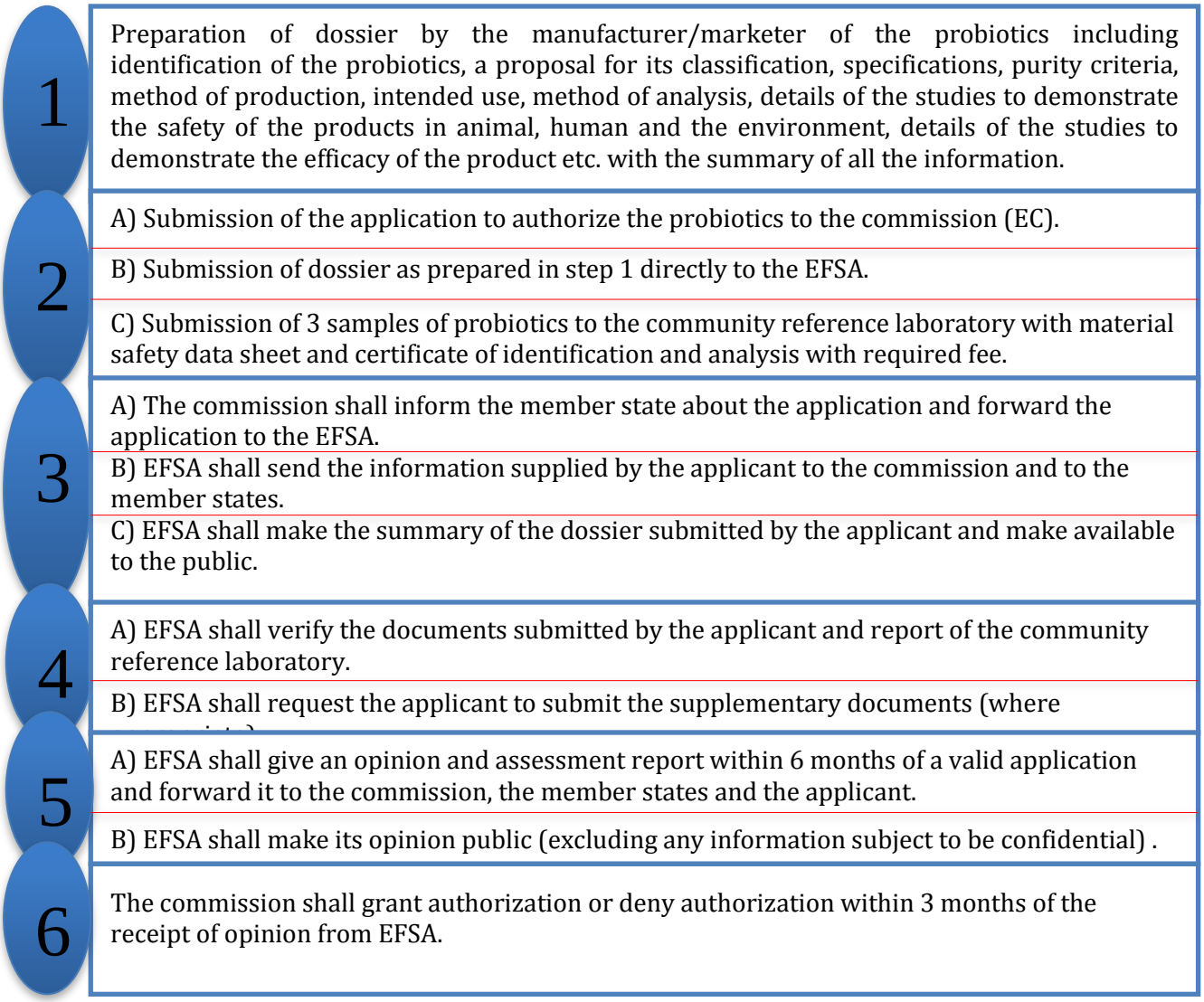

Figure S3: Pathway for the authorization of new probiotics in the European Union as per Regulations (EC) No. 1831/2003 and 429/2008.

9.4 Regulation of probiotic labelling

In the EU, Regulation (EC) No 1831/2003 on additives for use in animal nutrition covers labelling of probiotics. According to this legislation, it is illegal to sell feed additives (including probiotics) without clearly labelling the products with (a) specific name and functional group of the additives (b) name and address of the business responsible for the product (c) net weight or net volume (in case of liquid) (d) approval number to establish and operate the establishment or the intermediary pursuant (where appropriate) (e) instructions for use including the species and categories of animal (f) date of manufacture with batch number (Europen Commission, 2003). In addition to these general requirements for feed additives, probiotics should have the following specific information on their label: “the expiry date of the guarantee or the storage life from the date of manufacture, the directions for use, the strain identification number, and the number of colony-forming units per gram” (Europen Commission, 2003).

In 1987, a joint exercise by FDA, Association of American Feed Control Officials (AAFCO) and National Feed Ingredients Association (later merged with the American Feed Industry Association) consensually agreed to include the phrase “contains a source of live (viable), naturally occurring microorganisms” followed by the name of the microorganisms in the product with the content guarantee, as colony-forming units per gram on the label of commercial probiotic products (DFM) to be used in animal feed (Pendleton, 1998). Before this decision, probiotics had been labelled and regulated as commercial feed in accordance with the AAFCO regulations and the label had to contain guarantees for protein, fat and fibre, which was obviously not relevant to the product (Pendleton, 1998).

The classification and marketing of probiotics as feed additives in most countries, may result in the regulation and quality control of probiotics not being as stringent as that of veterinary drugs (Weese, 2003). This may result in probiotic labelling errors being overlooked.

Confusion and ambiguity prevails with regard to the regulation of probiotics in most countries. Approaches of risk assessment and level of stringency to authorize novel probiotics varies among nations. A global approach and guidelines to classify and regulate probiotics and assess risk could be effective in harmonizing regulations and protect public health.

10 Conclusion

Increasing intensification of animal agriculture with consequent imprudent use of antibiotic growth promoters poses risks to human and animal health in terms of increasing antibiotic resistance in pathogenic microorganisms. Live microorganisms have been studied and used as probiotics for a long time, and as an alternative to antibiotic growth promoters in animal production. Several probiotics have been found effective in improving animal performance and preventing disease and the spread of the enteric pathogens in both monogastric and ruminant livestock industries.

With the advancement in knowledge in gastrointestinal microbial ecology and mode of action of probiotics, the number of probiotic products available for use in animal nutrition is increasing. However, the microorganisms used as probiotics and their efficacy are highly variable. There are many promising effects of probiotics on animal performance and health. However, the major limitation for the widespread and sustainable use of probiotics is the uncertainty in the reproducibility of effect, with a wide range of probiotic species, livestock species and husbandry practice highlighting the complexity of the interactions in animal production systems. Study about the effects of a particular microbial strain on variety of animal species, age groups, growth condition, diet types may help to identify the condition in which the probiotics could work.

Although the use of probiotics could be a potentially viable solution to address the issue of increasing antibiotic resistance, it requires much further study on the effect, mechanism of action and safety of probiotics, to obtain consistent effects and a similar economic benefit to AGPs.

The claims made by commercial probiotic producers are often difficult to substantiate due to variation in animal species and husbandry practices and lack of scientific publications regarding the product. It is not possible to generalize the mechanism of action of probiotics. As the effects of probiotics in host is the outcome of interaction between the host and the probiotic microorganism, further studies should be focussed on host-probiotic interactions to elucidate the mode of action. Although generally considered safe, there is little evidence that probiotics are absolutely safe and it has been agreed that "zero risk does not exist" (Marteau, 2001). Therefore, uncertainty would always exist about the efficacy and safety of probiotics. Studies about the minimum required dose of particular probiotic to achieve intended benefits and maximum dose rate which could be used without any adverse effects on host help to assure the benefits and minimize the risk.

Further studies are also required to determine whether the probiotics used in animal nutrition enter the human food chain and how they affect human health. Information about specific precautions about handling by particularly vulnerable populations like immunocompromised people or use in such host may further help to reduce the risk.

The stringencies of the regulations on the use of probiotics in animal agriculture vary even in developed countries. Regulation of probiotics in the EU based on the assessment by a scientific committee of experts reviewing identity, safety and efficacy of the probiotic microorganisms is exemplary.

The issue of maintaining safety and efficacy of probiotics could be more serious in developing countries where institutions that can do research on the efficacy of such probiotics and regulate the proper use of probiotics, are often in need of strengthening and capacity building. Therefore, focussing on relevant research for identification of risk associated with probiotics together with capacity building of competent regulation authority are important aspects to protect public health and animal health.

Bacterial genera commonly used as probiotics have been found to harbour antibiotic resistance genes on mobile genetic elements capable of transferring to potential enteric pathogens. Using microbial strain as probiotics only with proven absence of transferable antibiotic resistance genes could minimize this serious safety risk. Similar precautions should be carried out while using microbes with acquired resistance genes.

Therefore, international guidelines for the production, marketing and use of probiotics in animal nutrition are essential, especially with increasing globalisation. Such guidelines would help prevent the use of inappropriate microorganisms as probiotics and maintain the efficacy of probiotics in achieving the targeted benefits. Such guidelines would assist institutions involved in the production, marketing and regulation of probiotics and protect public health. Such guidelines should also give detailed instructions for the analysis of the risk associated with probiotics intended for use in animal production.

Appendix 2. Supplementary material for chapter 4

Table S9: Sequencing run statistics of experiment no. 1

Sample ID	Sample	Treatment	Total Read Count R1	Total Read Count R2	Reads Passing QC R1	Reads Passing QC R2	QIIME pre-filtered reads R1	QIIME unclustered singletons R1	Reads in complete OTU table R1	Reads in filtered OTU table (>0.05% abundance) R1
S1764	Ileum	H57	65966	65966	42180	7156	92	698	41390	25891
S1771	Ileum	H57	96588	96588	59656	17040	388	144	59124	52794
S1784	Ileum	H57	113755	113755	83704	28025	78	203	83423	75773
S1765	Ileum	H57	182870	182870	112721	31412	1766	2319	108636	51165
S1785	Ileum	H57	81966	81966	59232	14349	1551	148	57533	50240
S1795	Ileum	H57	115362	115362	77258	23049	412	1487	75359	40234
S1792	Caecum	H57	84274	84274	57929	20316	517	98	57314	52835
S1788	Caecum	H57	121120	121120	86942	31500	11	332	86599	68976
S1770	Caecum	H57	100191	100191	72984	17866	219	53	72712	70357
S1781	Caecum	H57	98296	98296	66184	23757	21	163	66000	58470
S1793	Caecum	H57	138393	138393	90063	26941	577	1652	87834	47106
S1761	Caecum	H57	129071	129071	85945	17827	522	1030	84393	48317
S1751	Caecum	H57	115231	115231	86627	26181	116	90	86421	81685
S1798	Caecum	H57	81035	81035	54295	17919	637	478	53180	34184
S1780	Caecum	H57	172318	172318	107380	29135	2213	1746	103421	54502
S1759	Caecum	H57	135814	135814	86075	20782	1136	1338	83601	45902
S1758	Caecum	H57	115493	115493	75438	17122	450	1016	73972	45136
S1757	Caecum	H57	88403	88403	56997	10696	210	902	55885	36527
S1794	Ileum	H57	140505	140505	94779	26490	299	1757	92723	47674
S1774	Ileum	H57	81890	81890	56259	22055	16	122	56121	51414
S1782	Ileum	H57	140216	140216	95855	35985	21	438	95396	79700
S1768	Ileum	H57	128271	128271	83956	16667	286	1468	82202	47034
S1791	Ileum	H57	75298	75298	52836	18208	3753	123	48960	43748
S1783	Ileum	H57	104783	104783	75359	26353	51	245	75063	62401

S1753	Caecum	Control	97139	97139	69043	20260	1143	178	67722	61454
S1796	Caecum	Control	104729	104729	71488	15401	7080	1166	63242	34442
S1786	Caecum	Control	83491	83491	60640	21431	2206	82	58352	54777
S1772	Caecum	Control	105891	105891	71298	24378	67	168	71063	61686
S1752	Caecum	Control	104227	104227	72700	19941	96	568	72036	56100
S1773	Caecum	Control	96034	96034	67921	13703	108	84	67729	63710
S1763	Ileum	Control	102443	102443	69716	7870	159	1478	68079	41360
S1766	Ileum	Control	130945	130945	85077	18049	547	1333	83197	47202
S1769	Ileum	Control	117869	117869	87131	20663	72	115	86944	80776
S1776	Ileum	Control	132003	132003	78061	18570	757	1415	75889	38931
S1760	Ileum	Control	146685	146685	98824	19408	380	2147	96297	49197
S1789	Ileum	Control	101975	101975	74215	30544	46	111	74058	68498
S1778	Ileum	Control	142096	142096	90255	21571	478	1827	87950	41495
S1754	Ileum	Control	110737	110737	80898	23002	128	120	80650	74719
S1779	Ileum	Control	168482	168482	103108	29630	629	2369	100110	51776
S1787	Ileum	Control	104218	104218	72477	25187	32	168	72277	62973
S1762	Ileum	Control	41466	41466	15817	126	31	371	15415	9090
S1755	Ileum	Control	70323	70323	49070	18027	30	60	48980	46127
S1777	Caecum	Control	126590	126590	78472	24000	872	1505	76095	37021
S1790	Caecum	Control	97265	97265	71295	28067	5	124	71166	62160
S1756	Caecum	Control	103780	103780	75248	20232	685	98	74465	69172
S1797	Caecum	Control	133700	133700	91181	18695	621	1278	89282	47274
S1775	Caecum	Control	186324	186324	105430	33716	4719	1510	99201	55362
S1767	Caecum	Control	115024	115024	73199	17002	575	1380	71244	40093
Average			113136	113136	75692	21173	767	786	74140	52655

Table S10: Sequencing run statistics of experiment no. 2

Sample	Sample	Treatment	Location	Total Read Count R1	Total Read Count R2	Reads Passing QC R1	Reads Passing QC R2	QIIME pre-filtered reads R1	QIIME unclustered singletons R1	Reads in complete OTU table R1	Reads in filtered OTU table (>0.05% abundance) R1
SA1539	Ileum	Control	Pen	10636	10636	9221	5540	1	65	9155	6346
SA1540	Ileum	Control	Pen	11433	11433	10142	5610	13	10	10119	9747
SA1541	Ileum	Control	Pen	14098	14098	12407	6451	5	65	12337	9917
SA1542	Ileum	Control	Pen	11085	11085	9715	5115	1	11	9703	9009
SA1543	Ileum	Control	Pen	13012	13012	11451	5911	1	8	11442	10924
SA1544	Ileum	Control	Pen	15048	15048	12006	6406	5	33	11968	11365
SA1545	Ileum	Control	Cage	11572	11572	10112	5749	2	7	10103	9541
SA1546	Ileum	Control	Cage	12916	12916	10944	6120	20	56	10868	9679
SA1547	Ileum	Control	Cage	10472	10472	9522	5211	0	9	9513	9324
SA1548	Ileum	Control	Cage	12728	12728	11394	6540	0	2	11392	11198
SA1549	Ileum	Control	Cage	10779	10779	9710	4722	21	2	9687	9440
SA1550	Ileum	Control	Cage	20071	20071	18120	9147	2	11	18107	18031
SA1551	Ileum	H57	Pen	10254	10254	8872	4887	0	40	8832	5682
SA1552	Ileum	H57	Pen	18850	18850	16611	9417	4	11	16596	16322
SA1553	Ileum	H57	Pen	12140	12140	10861	5946	0	23	10838	10054
SA1554	Ileum	H57	Pen	14601	14601	12622	7112	77	107	12438	7750
SA1555	Ileum	H57	Pen	15619	15619	13856	7493	0	15	13841	12978
SA1556	Ileum	H57	Pen	8227	8227	7052	4266	1	35	7016	4910
SA1557	Ileum	H57	Cage	10514	10514	9430	4580	4	18	9408	9114
SA1558	Ileum	H57	Cage	25504	25504	22672	10353	0	8	22664	21403
SA1559	Ileum	H57	Cage	8788	8788	7826	3944	2	9	7815	7323
SA1560	Ileum	H57	Cage	15015	15015	12870	6557	9	47	12814	10494
SA1561	Ileum	H57	Cage	12402	12402	11078	5549	2	10	11066	10495
SA1562	Ileum	H57	Cage	10954	10954	9646	4568	2	20	9624	8657
SA1563	Caeca	Control	Pen	9118	9118	7848	4795	7	129	7712	4435
SA1564	Caeca	Control	Pen	10079	10079	8618	5313	12	206	8400	3525

SA1565	Caeca	Control	Pen	11560	11560	8442	2460	8	157	8277	3846
SA1566	Caeca	Control	Pen	11688	11688	10112	5641	2	131	9979	5841
SA1567	Caeca	Control	Pen	8023	8023	3835	111	2	143	3690	1810
SA1568	Caeca	Control	Pen	14782	14782	12187	7323	3	364	11820	4237
SA1569	Caeca	Control	Cage	48928	48928	41212	25833	44	291	40877	24512
SA1570	Caeca	Control	Cage	10777	10777	8883	5369	23	161	8699	4418
SA1571	Caeca	Control	Cage	7382	7382	6422	3659	6	72	6344	3127
SA1572	Caeca	Control	Cage	9284	9284	7859	4930	5	65	7789	4710
SA1573	Caeca	Control	Cage	11093	11093	9333	4546	7	183	9143	4771
SA1574	Caeca	Control	Cage	14440	14440	12384	7313	8	108	12268	6599
SA1575	Caeca	H57	Pen	11598	11598	9848	4230	16	201	9631	4324
SA1576	Caeca	H57	Pen	13966	13966	11989	7288	6	255	11728	6546
SA1577	Caeca	H57	Pen	23002	23002	18826	11155	6	354	18466	9883
SA1578	Caeca	H57	Pen	13268	13268	10890	5259	4	219	10667	5803
SA1579	Caeca	H57	Pen	9876	9876	8274	4676	5	107	8162	5259
SA1580	Caeca	H57	Pen	8607	8607	7240	4210	5	161	7074	3811
SA1581	Caeca	H57	Cage	8678	8678	7447	3974	7	81	7359	3892
SA1582	Caeca	H57	Cage	464	464	377	184	2	6	369	174
SA1583	Caeca	H57	Cage	12125	12125	10165	5296	10	122	10033	5299
SA1584	Caeca	H57	Cage	10575	10575	8336	4482	12	235	8089	2972
SA1585	Caeca	H57	Cage	12890	12890	10865	5838	5	169	10691	6329
SA1586	Caeca	H57	Cage	11308	11308	9472	4923	3	194	9275	5500
Average				12921	12921	11021	5958	8	99	10914	7944

Table S11: Sequencing run statistics of experiment no. 3

Sample ID	Age	Diet	Sample	Treatment	Total Read Count R1	Total Read Count R2	Reads Passing QC R1	Reads Passing QC R2	QIIME pre-filtered reads R1	QIIME unclustered singletons R1	Reads in complete OTU table R1	Reads in filtered OTU table (>0.05% abundance) R1
SA1587	4	Sorghum	Ileum	Control	21389	21389	19096	13029	3	14	19079	18809
SA1588	4	Sorghum	Ileum	Control	19357	19357	16996	11653	0	17	16979	15314
SA1589	4	Sorghum	Ileum	Control	12731	12731	11306	6593	7	7	11292	10680
SA1590	4	Sorghum	Ileum	Control	8240	8240	7302	4628	8	11	7283	6646
SA1591	4	Sorghum	Ileum	Control	12304	12304	10692	6956	0	23	10669	9458
SA1592	4	Sorghum	Ileum	Control	13870	13870	11837	7890	4	23	11810	11184
SA1593	4	Sorghum	Ileum	H57	14012	14012	12385	7998	0	25	12360	11450
SA1594	4	Sorghum	Ileum	H57	11295	11295	9906	6525	9	9	9888	9491
SA1595	4	Sorghum	Ileum	H57	11293	11293	9741	6802	30	13	9698	9408
SA1596	4	Sorghum	Ileum	H57	8903	8903	7916	4807	4	1	7911	7638
SA1597	4	Sorghum	Ileum	H57	11663	11663	10500	6135	3	8	10489	10025
SA1598	4	Sorghum	Ileum	H57	22399	22399	19961	12519	6	9	19946	19326
SA1599	4	Sorghum	Caeca	Control	19124	19124	16427	8636	9	95	16323	10310
SA1600	4	Sorghum	Caeca	Control	13779	13779	11160	5905	13	109	11038	6209
SA1601	4	Sorghum	Caeca	Control	9479	9479	8106	4310	35	71	8000	4068
SA1602	4	Sorghum	Caeca	Control	8705	8705	7174	3732	8	102	7064	3728
SA1603	4	Sorghum	Caeca	Control	9533	9533	7749	4563	3	84	7662	3352
SA1604	4	Sorghum	Caeca	Control	13091	13091	10776	5248	36	79	10661	5901
SA1605	4	Sorghum	Caeca	H57	21093	21093	17998	9694	34	149	17815	11039
SA1606	4	Sorghum	Caeca	H57	12634	12634	10763	5914	4	43	10716	7879
SA1607	4	Sorghum	Caeca	H57	9536	9536	8027	4414	9	53	7965	5287
SA1608	4	Sorghum	Caeca	H57	13034	13034	10608	5882	3	126	10479	6158
SA1609	4	Sorghum	Caeca	H57	9112	9112	7640	4251	8	35	7597	5308
SA1610	4	Sorghum	Caeca	H57	11225	11225	9135	4997	29	50	9056	5810
SA1611	4	Wheat	Ileum	Control	13979	13979	12254	7181	0	11	12243	11708
SA1612	4	Wheat	Ileum	Control	13537	13537	11328	5966	1	13	11314	10956

SA1613	4	Wheat	Ileum	Control	12898	12898	11532	6843	4	13	11515	11017
SA1614	4	Wheat	Ileum	Control	18009	18009	15471	9105	62	26	15383	13742
SA1615	4	Wheat	Ileum	Control	13645	13645	12140	7291	1	28	12111	11890
SA1616	4	Wheat	Ileum	Control	15098	15098	12816	8019	192	9	12615	11880
SA1617	4	Wheat	Ileum	H57	12447	12447	10697	6722	1	11	10685	10306
SA1618	4	Wheat	Ileum	H57	8914	8914	6296	3374	9	17	6270	5841
SA1619	4	Wheat	Ileum	H57	11745	11745	10578	6985	7	11	10560	10359
SA1620	4	Wheat	Ileum	H57	12649	12649	11105	7714	7	4	11094	10584
SA1621	4	Wheat	Ileum	H57	7977	7977	7148	4388	14	15	7119	6734
SA1622	4	Wheat	Ileum	H57	9621	9621	8425	5039	7	6	8412	8161
SA1623	4	Wheat	Caeca	Control	9820	9820	8399	4265	14	95	8290	4563
SA1624	4	Wheat	Caeca	Control	8689	8689	6953	4204	1	36	6916	4941
SA1625	4	Wheat	Caeca	Control	20483	20483	16703	9936	7	85	16611	10908
SA1626	4	Wheat	Caeca	Control	11589	11589	9493	5251	22	49	9422	5719
SA1627	4	Wheat	Caeca	Control	13748	13748	11967	6931	38	33	11896	8925
SA1628	4	Wheat	Caeca	Control	11573	11573	9902	5860	11	34	9857	6933
SA1629	4	Wheat	Caeca	H57	13592	13592	11803	6565	12	41	11750	8520
SA1630	4	Wheat	Caeca	H57	11721	11721	10142	5407	5	57	10080	7130
SA1631	4	Wheat	Caeca	H57	12449	12449	10685	5917	20	49	10616	7738
SA1632	4	Wheat	Caeca	H57	32295	32295	26277	15984	16	107	26154	18015
SA1633	4	Wheat	Caeca	H57	15382	15382	13204	7387	20	55	13129	8732
SA1634	4	Wheat	Caeca	H57	12927	12927	10946	5921	4	86	10856	5767
SA1635	4	Blend	Ileum	Control	25441	25441	22785	13574	8	5	22772	22281
SA1636	4	Blend	Ileum	Control	14232	14232	12656	8491	7	8	12641	12237
SA1637	4	Blend	Ileum	Control	8363	8363	7460	4837	5	10	7445	7142
SA1638	4	Blend	Ileum	Control	16818	16818	14876	8941	2	13	14861	14029
SA1639	4	Blend	Ileum	Control	10300	10300	9131	5845	21	4	9106	8633
SA1640	4	Blend	Ileum	Control	28331	28331	24792	16222	12	23	24757	22644
SA1641	4	Blend	Ileum	H57	18390	18390	16402	10526	2	7	16393	15587
SA1642	4	Blend	Ileum	H57	14663	14663	12718	7889	11	14	12693	11765
SA1643	4	Blend	Ileum	H57	11968	11968	10738	6763	10	6	10722	10295

SA1644	4	Blend	Ileum	H57	39460	39460	34508	20682	113	19	34376	33414
SA1645	4	Blend	Ileum	H57	16343	16343	14663	8333	10	18	14635	14106
SA1646	4	Blend	Ileum	H57	15166	15166	13472	7787	9	10	13453	13047
SA1647	4	Blend	Caeca	Control	21028	21028	18465	9561	24	89	18352	11414
SA1648	4	Blend	Caeca	Control	20065	20065	16814	9241	9	86	16719	11530
SA1649	4	Blend	Caeca	Control	30220	30220	23527	12045	19	151	23357	11987
SA1650	4	Blend	Caeca	Control	11115	11115	6785	1148	4	52	6729	4443
SA1651	4	Blend	Caeca	Control	19709	19709	16633	8995	22	66	16545	11342
SA1652	4	Blend	Caeca	Control	14201	14201	11688	7100	40	119	11529	6922
SA1653	4	Blend	Caeca	H57	11059	11059	9714	4988	20	58	9636	5977
SA1654	4	Blend	Caeca	H57	15496	15496	12682	6869	13	105	12564	6750
SA1655	4	Blend	Caeca	H57	16040	16040	13935	6906	63	53	13819	9835
SA1656	4	Blend	Caeca	H57	14460	14460	11628	6535	33	69	11526	6676
SA1657	4	Blend	Caeca	H57	24784	24784	20065	8990	5	140	19920	10856
SA1658	4	Blend	Caeca	H57	9845	9845	8143	4643	15	44	8084	5248
SA1659	13	Sorghum	Ileum	Control	36330	36330	31973	20293	166	35	31772	29220
SA1660	13	Sorghum	Ileum	Control	9055	9055	7928	4887	50	4	7874	7656
SA1661	13	Sorghum	Ileum	Control	37613	37613	33640	19109	67	34	33539	31994
SA1662	13	Sorghum	Ileum	Control	14272	14272	12628	7236	97	10	12521	12150
SA1663	13	Sorghum	Ileum	Control	14426	14426	12867	7386	2	4	12861	12604
SA1664	13	Sorghum	Ileum	Control	24346	24346	20841	14113	18	17	20806	20258
SA1665	13	Sorghum	Ileum	H57	14196	14196	12639	7389	10	14	12615	12386
SA1666	13	Sorghum	Ileum	H57	23997	23997	18786	11696	77	36	18673	17235
SA1667	13	Sorghum	Ileum	H57	14535	14535	13003	7986	11	13	12979	12347
SA1668	13	Sorghum	Ileum	H57	27383	27383	23663	15569	1432	13	22218	21197
SA1669	13	Sorghum	Ileum	H57	14456	14456	12856	6917	8	8	12840	12356
SA1670	13	Sorghum	Ileum	H57	26570	26570	23041	13645	355	35	22651	20937
SA1671	13	Sorghum	Caeca	Control	12564	12564	10581	5108	30	30	10521	8000
SA1672	13	Sorghum	Caeca	Control	13016	13016	9989	5219	17	141	9831	5219
SA1673	13	Sorghum	Caeca	Control	11402	11402	9564	6051	11	53	9500	6046
SA1674	13	Sorghum	Caeca	Control	11629	11629	9502	5492	10	28	9464	6568

SA1675	13	Sorghum	Caeca	Control	13765	13765	11358	5965	20	87	11251	7764
SA1676	13	Sorghum	Caeca	Control	11326	11326	9434	4757	6	69	9359	5395
SA1677	13	Sorghum	Caeca	H57	11449	11449	9864	4669	10	46	9808	6299
SA1678	13	Sorghum	Caeca	H57	18148	18148	14932	7399	19	54	14859	9998
SA1679	13	Sorghum	Caeca	H57	12757	12757	10745	5549	9	15	10721	6585
SA1680	13	Sorghum	Caeca	H57	32361	32361	26572	16581	21	118	26433	17987
SA1681	13	Sorghum	Caeca	H57	11514	11514	9678	5534	24	99	9555	5304
SA1682	13	Sorghum	Caeca	H57	22339	22339	18408	10455	13	52	18343	13574
SA1683	13	Wheat	Ileum	Control	17142	17142	15217	9618	10	12	15195	14094
SA1684	13	Wheat	Ileum	Control	26171	26171	23314	15174	22	24	23268	22899
SA1685	13	Wheat	Ileum	Control	24217	24217	21617	12594	15	14	21588	20921
SA1686	13	Wheat	Ileum	Control	10427	10427	9218	5376	32	6	9180	8939
SA1687	13	Wheat	Ileum	Control	15790	15790	13896	8467	85	14	13797	12615
SA1688	13	Wheat	Ileum	Control	18043	18043	15427	10357	75	22	15330	14142
SA1689	13	Wheat	Ileum	H57	19467	19467	17184	10638	9	16	17159	16275
SA1690	13	Wheat	Ileum	H57	19917	19917	17324	10279	77	24	17223	15900
SA1691	13	Wheat	Ileum	H57	14498	14498	13037	8194	60	4	12973	12459
SA1692	13	Wheat	Ileum	H57	8366	8366	7495	4771	10	9	7476	7038
SA1693	13	Wheat	Ileum	H57	25604	25604	22956	13323	80	32	22844	21122
SA1694	13	Wheat	Ileum	H57	22961	22961	20356	12241	181	15	20160	18652
SA1695	13	Wheat	Caeca	Control	17582	17582	15116	7214	31	25	15060	11029
SA1696	13	Wheat	Caeca	Control	15913	15913	13229	7033	8	48	13173	10151
SA1697	13	Wheat	Caeca	Control	14459	14459	12397	6988	9	35	12353	8730
SA1698	13	Wheat	Caeca	Control	17416	17416	14684	8476	19	41	14624	10904
SA1699	13	Wheat	Caeca	Control	20737	20737	17471	10331	16	60	17395	10761
SA1700	13	Wheat	Caeca	Control	16319	16319	13709	7438	48	109	13552	7524
SA1701	13	Wheat	Caeca	H57	19812	19812	16850	8347	74	39	16737	13639
SA1702	13	Wheat	Caeca	H57	13464	13464	11265	5886	15	48	11202	7719
SA1703	13	Wheat	Caeca	H57	16686	16686	13919	6389	13	35	13871	10821
SA1704	13	Wheat	Caeca	H57	11450	11450	9193	5709	12	37	9144	6364
SA1705	13	Wheat	Caeca	H57	9927	9927	8434	4622	15	89	8330	5383

SA1706	13	Wheat	Caeca	H57	15552	15552	12924	6853	10	105	12809	8157
SA1707	13	Blend	Ileum	Control	16641	16641	14480	9738	39	12	14429	13541
SA1708	13	Blend	Ileum	Control	8049	8049	6918	4474	50	12	6856	6719
SA1709	13	Blend	Ileum	Control	22781	22781	20171	11692	18	10	20143	19645
SA1710	13	Blend	Ileum	Control	9624	9624	8414	5209	18	8	8388	7806
SA1711	13	Blend	Ileum	Control	25701	25701	22767	13080	3	13	22751	21625
SA1712	13	Blend	Ileum	Control	17694	17694	14644	9584	27	15	14602	13644
SA1713	13	Blend	Ileum	H57	9753	9753	8636	5294	12	3	8621	8143
SA1714	13	Blend	Ileum	H57	17742	17742	15316	9392	20	6	15290	14717
SA1715	13	Blend	Ileum	H57	19165	19165	16964	10837	12	13	16939	15452
SA1716	13	Blend	Ileum	H57	17584	17584	15546	10102	10	20	15516	14510
SA1717	13	Blend	Ileum	H57	18581	18581	16581	9904	43	13	16525	15738
SA1718	13	Blend	Ileum	H57	21932	21932	16367	9911	18	15	16334	15465
SA1719	13	Blend	Caeca	Control	20593	20593	17501	9873	42	66	17393	12070
SA1720	13	Blend	Caeca	Control	28630	28630	23372	13752	29	64	23279	16929
SA1721	13	Blend	Caeca	Control	13747	13747	11818	7162	5	68	11745	8099
SA1722	13	Blend	Caeca	Control	24487	24487	20540	11905	9	136	20395	12245
SA1723	13	Blend	Caeca	Control	23087	23087	19870	11878	17	157	19696	11481
SA1724	13	Blend	Caeca	Control	34783	34783	29585	18569	21	143	29421	21059
SA1725	13	Blend	Caeca	H57	33653	33653	28985	12724	37	113	28835	19564
SA1726	13	Blend	Caeca	H57	15007	15007	12933	7873	2	19	12912	10357
SA1727	13	Blend	Caeca	H57	20495	20495	17042	7227	3	89	16950	9786
SA1728	13	Blend	Caeca	H57	25934	25934	21803	12838	13	129	21661	11547
SA1729	13	Blend	Caeca	H57	14133	14133	12023	6882	9	94	11920	7170
SA1730	13	Blend	Caeca	H57	15557	15557	13108	7443	49	159	12900	7093
SA1731	21	Sorghum	Ileum	Control	71719	71719	61148	26812	171	99	60878	51945
SA1732	21	Sorghum	Ileum	Control	66077	66077	56906	34567	332	45	56529	54729
SA1733	21	Sorghum	Ileum	Control	75039	75039	64608	38415	1260	56	63292	60430
SA1734	21	Sorghum	Ileum	Control	57216	57216	49798	26338	10	53	49735	48326
SA1735	21	Sorghum	Ileum	Control	55527	55527	48022	27566	2904	40	45078	42384
SA1736	21	Sorghum	Ileum	Control	68555	68555	59457	34548	873	249	58335	54198

SA1737	21	Sorghum	Ileum	H57	67051	67051	55003	26740	70	77	54856	49099
SA1738	21	Sorghum	Ileum	H57	69075	69075	60175	35161	399	73	59703	54227
SA1739	21	Sorghum	Ileum	H57	69980	69980	61465	38344	51	113	61301	53709
SA1740	21	Sorghum	Ileum	H57	33748	33748	29941	18855	157	23	29761	29002
SA1741	21	Sorghum	Ileum	H57	52926	52926	47374	27658	32	22	47320	46415
SA1742	21	Sorghum	Ileum	H57	109446	109446	97860	53342	119	62	97679	93375
SA1743	21	Sorghum	Caeca	Control	79067	79067	67905	36048	66	1015	66824	39099
SA1744	21	Sorghum	Caeca	Control	84883	84883	70857	32492	85	761	70011	42811
SA1745	21	Sorghum	Caeca	Control	83935	83935	69658	30525	248	623	68787	44628
SA1746	21	Sorghum	Caeca	Control	73293	73293	61357	31549	172	671	60514	36281
SA1747	21	Sorghum	Caeca	Control	73716	73716	61659	29538	363	679	60617	34217
SA1748	21	Sorghum	Caeca	Control	124517	124517	102181	42761	423	1668	100090	56012
SA1749	21	Sorghum	Caeca	H57	42045	42045	35566	16625	89	728	34749	19213
SA1750	21	Sorghum	Caeca	H57	48926	48926	41146	19073	74	433	40639	22134
SA1751	21	Sorghum	Caeca	H57	65247	65247	56110	28481	199	299	55612	41233
SA1752	21	Sorghum	Caeca	H57	75177	75177	63004	27823	54	837	62113	37366
SA1753	21	Sorghum	Caeca	H57	48666	48666	38945	16690	27	766	38152	21806
SA1754	21	Sorghum	Caeca	H57	71234	71234	59905	30196	93	852	58960	33367
SA1755	21	Wheat	Ileum	Control	61530	61530	54041	33098	159	37	53845	52276
SA1756	21	Wheat	Ileum	Control	64733	64733	57319	34901	89	63	57167	54179
SA1757	21	Wheat	Ileum	Control	78782	78782	65172	41006	28	43	65101	63388
SA1758	21	Wheat	Ileum	Control	48967	48967	42626	24513	110	60	42456	39771
SA1759	21	Wheat	Ileum	Control	40745	40745	36440	22916	244	49	36147	33288
SA1760	21	Wheat	Ileum	Control	72208	72208	63879	37877	1753	142	61984	56165
SA1761	21	Wheat	Ileum	H57	73056	73056	63971	40345	48	56	63867	59535
SA1762	21	Wheat	Ileum	H57	61830	61830	54838	33113	15	48	54775	52810
SA1763	21	Wheat	Ileum	H57	53771	53771	48226	28960	98	17	48111	46741
SA1764	21	Wheat	Ileum	H57	77433	77433	68844	43582	525	121	68198	59660
SA1765	21	Wheat	Ileum	H57	60729	60729	54711	28875	574	31	54106	51988
SA1766	21	Wheat	Ileum	H57	82778	82778	73684	43492	10	65	73609	68845
SA1767	21	Wheat	Caeca	Control	67825	67825	57438	29279	46	1779	55613	26886

SA1768	21	Wheat	Caeca	Control	42526	42526	35623	17076	39	438	35146	21617
SA1769	21	Wheat	Caeca	Control	59084	59084	48228	28264	25	517	47686	29188
SA1770	21	Wheat	Caeca	Control	71122	71122	60254	34217	38	483	59733	36101
SA1771	21	Wheat	Caeca	Control	61075	61075	48598	15895	43	575	47980	31117
SA1772	21	Wheat	Caeca	Control	66968	66968	52176	23990	58	1205	50913	23809
SA1773	21	Wheat	Caeca	H57	49050	49050	40107	18696	22	643	39442	22329
SA1774	21	Wheat	Caeca	H57	40901	40901	33729	14644	32	466	33231	20048
SA1775	21	Wheat	Caeca	H57	63295	63295	53343	24414	32	1048	52263	29644
SA1776	21	Wheat	Caeca	H57	75176	75176	62916	32487	99	516	62301	41097
SA1777	21	Wheat	Caeca	H57	68086	68086	55958	26080	72	478	55408	35824
SA1778	21	Wheat	Caeca	H57	69435	69435	58081	31154	37	595	57449	36535
SA1779	21	Blend	Ileum	Control	125120	125120	110080	67561	246	101	109733	103301
SA1780	21	Blend	Ileum	Control	65276	65276	57494	35300	150	52	57292	54993
SA1781	21	Blend	Ileum	Control	65755	65755	56810	35318	114	28	56668	55693
SA1782	21	Blend	Ileum	Control	62633	62633	54607	31612	734	68	53805	50549
SA1783	21	Blend	Ileum	Control	67759	67759	60758	38800	479	60	60219	57955
SA1784	21	Blend	Ileum	Control	66901	66901	59259	36354	95	39	59125	56643
SA1785	21	Blend	Ileum	H57	60142	60142	52673	31931	44	54	52575	50967
SA1786	21	Blend	Ileum	H57	44499	44499	39297	23662	51	39	39207	37699
SA1787	21	Blend	Ileum	H57	54442	54442	48748	26251	33	26	48689	45908
SA1788	21	Blend	Ileum	H57	62627	62627	55461	32213	204	68	55189	51651
SA1789	21	Blend	Ileum	H57	165809	165809	147189	80205	330	52	146807	139080
SA1790	21	Blend	Ileum	H57	112227	112227	99073	53918	4599	69	94405	91054
SA1791	21	Blend	Caeca	Control	210896	210896	177591	82517	225	2338	175028	103327
SA1792	21	Blend	Caeca	Control	60023	60023	49703	23712	47	842	48814	27601
SA1793	21	Blend	Caeca	Control	81683	81683	66966	26232	23	1025	65918	38402
SA1794	21	Blend	Caeca	Control	60834	60834	50986	25138	70	549	50367	31113
SA1795	21	Blend	Caeca	Control	95710	95710	77876	39622	91	1354	76431	35215
SA1796	21	Blend	Caeca	Control	55078	55078	43626	23477	46	519	43061	28993
SA1797	21	Blend	Caeca	H57	45999	45999	38073	18730	110	443	37520	22073
SA1798	21	Blend	Caeca	H57	84125	84125	67283	29953	77	1091	66115	28234

SA1799	21	Blend	Caeca	H57	64672	64672	54716	27439	71	940	53705	32880
SA1800	21	Blend	Caeca	H57	47686	47686	39866	21702	32	763	39071	22493
SA1801	21	Blend	Caeca	H57	78730	78730	65052	30680	41	2397	62614	30796
SA1802	21	Blend	Caeca	H57	61558	61558	50789	24881	26	1361	49402	26419
SA1803	13	Sorghum	Faeces	Control	65697	65697	57649	33647	325	38	57286	56230
SA1804	13	Sorghum	Faeces	Control	78298	78298	67978	41095	56	57	67865	65952
SA1805	13	Sorghum	Faeces	Control	70666	70666	60412	33592	70	1061	59281	33140
SA1806	13	Sorghum	Faeces	Control	40063	40063	35050	18283	74	127	34849	28573
SA1807	13	Sorghum	Faeces	Control	84117	84117	75392	43397	17	47	75328	73093
SA1808	13	Sorghum	Faeces	Control	85619	85619	75932	42389	51	56	75825	72887
SA1809	13	Sorghum	Faeces	H57	45205	45205	39699	23399	76	47	39576	37622
SA1810	13	Sorghum	Faeces	H57	50582	50582	44551	24754	48	48	44455	42121
SA1811	13	Sorghum	Faeces	H57	46311	46311	40250	24187	69	13	40168	39141
SA1812	13	Sorghum	Faeces	H57	37366	37366	31741	20510	21	16	31704	31170
SA1813	13	Sorghum	Faeces	H57	80490	80490	71855	40362	81	47	71727	69334
SA1814	13	Sorghum	Faeces	H57	112704	112704	95560	54498	54	67	95439	92366
SA1815	13	Wheat	Faeces	Control	59243	59243	53148	31645	9	35	53104	51880
SA1816	13	Wheat	Faeces	Control	74966	74966	63130	34729	23	517	62590	43452
SA1817	13	Wheat	Faeces	Control	50287	50287	40706	26348	9	32	40665	39972
SA1818	13	Wheat	Faeces	Control	59555	59555	49853	29023	12	49	49792	48036
SA1819	13	Wheat	Faeces	Control	47574	47574	41391	23909	1	63	41327	39539
SA1820	13	Wheat	Faeces	Control	64130	64130	55364	34157	10	215	55139	44014
SA1821	13	Wheat	Faeces	H57	32230	32230	28587	14622	18	16	28553	28053
SA1822	13	Wheat	Faeces	H57	29510	29510	25525	13281	6	51	25468	22504
SA1823	13	Wheat	Faeces	H57	12885	12885	11578	6794	1	4	11573	11481
SA1824	13	Wheat	Faeces	H57	32863	32863	29114	16331	1	16	29097	28134
SA1825	13	Wheat	Faeces	H57	56926	56926	50137	27761	23	50	50064	46754
SA1826	13	Wheat	Faeces	H57	44359	44359	37841	21080	689	57	37095	35167
SA1827	13	Blend	Faeces	Control	291699	291699	256263	159362	80	255	255928	241947
SA1828	13	Blend	Faeces	Control	275125	275125	239858	150741	750	355	238753	220996
SA1829	13	Blend	Faeces	Control	280254	280254	247844	150021	195	327	247322	227979

SA1830	13	Blend	Faeces	Control	199029	199029	169325	94792	743	322	168260	148755
SA1831	13	Blend	Faeces	Control	336112	336112	290732	178675	290	3651	286791	147651
SA1832	13	Blend	Faeces	Control	342343	342343	302473	185949	321	136	302016	297591
SA1833	13	Blend	Faeces	H57	359038	359038	306232	189355	1863	521	303848	266898
SA1834	13	Blend	Faeces	H57	318909	318909	277696	171870	174	240	277282	265906
SA1835	13	Blend	Faeces	H57	163465	163465	143539	85426	89	101	143349	139246
SA1836	13	Blend	Faeces	H57	133513	133513	116225	51566	62	87	116076	108145
SA1837	13	Blend	Faeces	H57	196575	196575	173133	97611	224	151	172758	165599
SA1838	13	Blend	Faeces	H57	160035	160035	141453	74552	164	124	141165	135913
Average					47194	47194	40600	22900	128	196	40276	33224

Appendix 3. Supplementary material for chapter 5

Table S12: Average relative abundance of functional genes at different level of KEGG Orthology (KO) classification in the caecum of chickens fed sorghum based and wheat based diet at the age of day 13

KO Classification			Average relative abundance			
Level 1	**Level 2**	**Level 3**	**Sorghum Control**	**Sorghum H57**	**Wheat Control**	**Wheat H57**
Cellular Processes	Cell communication	04530 Tight junction [PATH:ko04530]	0	0	0.000809	0
Cellular Processes	Cell growth and death	04110 Cell cycle [PATH:ko04110]	0	0.000142	0	0
Cellular Processes	Cell growth and death	04112 Cell cycle - Caulobacter [PATH:ko04112]	2.287779	2.321593	2.479505	1.877131
Cellular Processes	Cell growth and death	04113 Meiosis - yeast [PATH:ko04113]	0.000091	0	0.000200	0
Cellular Processes	Cell growth and death	04210 Apoptosis [PATH:ko04210]	0	0.003278	0.002357	0.012053
Cellular Processes	Cell motility	02030 Bacterial chemotaxis [PATH:ko02030]	0.280768	0.289414	0.360128	0.328790
Cellular Processes	Cell motility	02040 Flagellar assembly [PATH:ko02040]	0.032425	0.083631	0.015961	0.061261
Cellular Processes	Transport and catabolism	04142 Lysosome [PATH:ko04142]	0.384032	0.046664	0.036814	0.098756
Cellular Processes	Transport and catabolism	04144 Endocytosis [PATH:ko04144]	0.000878	0	0.000191	0.000087
Cellular Processes	Transport and catabolism	04145 Phagosome [PATH:ko04145]	0	0.000354	0.000809	0
Cellular Processes	Transport and catabolism	04146 Peroxisome [PATH:ko04146]	0.498239	0.243095	0.382283	0.295365
Environmental Information Processing	Membrane transport	02010 ABC transporters [PATH:ko02010]	6.261668	8.537975	8.998237	8.800924
Environmental Information Processing	Membrane transport	02060 Phosphotransferase system (PTS) [PATH:ko02060]	0.782979	1.511406	1.015719	1.441844
Environmental Information Processing	Membrane transport	03070 Bacterial secretion system [PATH:ko03070]	2.052348	2.201130	2.053015	2.152386
Environmental Information Processing	Signal transduction	02020 Two-component system [PATH:ko02020]	1.713799	1.560137	1.967743	1.736254
Environmental Information Processing	Signal transduction	04011 MAPK signaling pathway - yeast [PATH:ko04011]	0.021631	0.004340	0.003493	0.016080
Environmental Information Processing	Signal transduction	04020 Calcium signaling pathway [PATH:ko04020]	0.000102	0.000147	0	0
Environmental Information Processing	Signal transduction	04066 HIF-1 signaling pathway [PATH:ko04066]	0.566587	0.477539	0.421366	0.538426
Environmental Information Processing	Signal transduction	04070 Phosphatidylinositol signaling system [PATH:ko04070]	0.067782	0.018341	0.080829	0.066764

Environmental Information Processing	Signal transduction	04151 PI3K-Akt signaling pathway [PATH:ko04151]	0.194971	0.216140	0.121106	0.121625
Environmental Information Processing	Signal transduction	04310 Wnt signaling pathway [PATH:ko04310]	0	0.000337	0	0
Environmental Information Processing	Signaling molecules and interaction	04080 Neuroactive ligand-receptor interaction [PATH:ko04080]	0	0.000049	0	0
Environmental Information Processing	Signaling molecules and interaction	04512 ECM-receptor interaction [PATH:ko04512]	0	0	0	0.000089
Environmental Information Processing	Signaling molecules and interaction	04514 Cell adhesion molecules (CAMs) [PATH:ko04514]	0	0	0	0.000331
Genetic Information Processing	Folding, sorting and degradation	03018 RNA degradation [PATH:ko03018]	3.271246	3.051442	2.945860	3.156704
Genetic Information Processing	Folding, sorting and degradation	03050 Proteasome [PATH:ko03050]	0.000681	0	0.098736	0.048840
Genetic Information Processing	Folding, sorting and degradation	03060 Protein export [PATH:ko03060]	0.147704	0.012507	0.038075	0.041043
Genetic Information Processing	Folding, sorting and degradation	04120 Ubiquitin mediated proteolysis [PATH:ko04120]	0	0.000098	0	0
Genetic Information Processing	Folding, sorting and degradation	04122 Sulfur relay system [PATH:ko04122]	0.274571	0.379589	0.311536	0.390393
Genetic Information Processing	Folding, sorting and degradation	04141 Protein processing in endoplasmic reticulum [PATH:ko04141]	0.023779	0.001921	0.007145	0.008098
Genetic Information Processing	Replication and repair	03030 DNA replication [PATH:ko03030]	1.890663	2.103299	1.882236	1.777900
Genetic Information Processing	Replication and repair	03410 Base excision repair [PATH:ko03410]	0.521935	0.474688	0.438833	0.433062
Genetic Information Processing	Replication and repair	03420 Nucleotide excision repair [PATH:ko03420]	1.975357	2.093031	2.022562	2.047384
Genetic Information Processing	Replication and repair	03430 Mismatch repair [PATH:ko03430]	0.490717	0.484418	0.303812	0.328866
Genetic Information Processing	Replication and repair	03440 Homologous recombination [PATH:ko03440]	1.535587	1.482270	1.586034	1.372346
Genetic Information Processing	Transcription	03020 RNA polymerase [PATH:ko03020]	4.169055	4.450112	4.214214	3.994087
Genetic Information Processing	Transcription	03040 Spliceosome [PATH:ko03040]	0.000774	0	0.005034	0.007945
Genetic Information Processing	Translation	00970 Aminoacyl-tRNA biosynthesis [PATH:ko00970]	6.770924	7.342966	6.227955	7.415765
Genetic Information Processing	Translation	03010 Ribosome [PATH:ko03010]	4.032363	4.175597	4.288662	3.666576
Genetic Information	Translation	03013 RNA transport [PATH:ko03013]	0.127781	0.060408	0.067084	0.118098

Processing						
Human Diseases	Cancers	05200 Pathways in cancer [PATH:ko05200]	0.005259	0.016465	0.006181	0.038963
Human Diseases	Cancers	05203 Viral carcinogenesis [PATH:ko05203]	0.301854	0.360568	0.234889	0.257388
Human Diseases	Cancers	05204 Chemical carcinogenesis [PATH:ko05204]	0.000583	0.002111	0.002041	0.002072
Human Diseases	Cancers	05215 Prostate cancer [PATH:ko05215]	0.001916	0	0	0
Human Diseases	Cancers	05219 Bladder cancer [PATH:ko05219]	0.004964	0.014036	0.003869	0.007620
Human Diseases	Endocrine and metabolic diseases	04940 Type I diabetes mellitus [PATH:ko04940]	0.032342	0.001048	0.000382	0.008703
Human Diseases	Immune diseases	05340 Primary immunodeficiency [PATH:ko05340]	0.000518	0.006056	0.003818	0.025685
Human Diseases	Infectious diseases	05100 Bacterial invasion of epithelial cells [PATH:ko05100]	0.000790	0.021876	0.026654	0.030734
Human Diseases	Infectious diseases	05111 Vibrio cholerae pathogenic cycle [PATH:ko05111]	0.045902	0.011509	0.048147	0.048477
Human Diseases	Infectious diseases	05120 Epithelial cell signaling in Helicobacter pylori infection [PATH:ko05120]	0.313873	0.196639	0.172268	0.231696
Human Diseases	Infectious diseases	05132 Salmonella infection [PATH:ko05132]	0.017519	0	0	0.001906
Human Diseases	Infectious diseases	05134 Legionellosis [PATH:ko05134]	0.252367	0.075781	0.162755	0.210492
Human Diseases	Infectious diseases	05142 Chagas disease (American trypanosomiasis) [PATH:ko05142]	0	0.000108	0.086515	0.139472
Human Diseases	Infectious diseases	05143 African trypanosomiasis [PATH:ko05143]	0.001019	0.003393	0	0.003761
Human Diseases	Infectious diseases	05144 Malaria [PATH:ko05144]	0	0.000098	0	0
Human Diseases	Infectious diseases	05146 Amoebiasis [PATH:ko05146]	0.013313	0.000501	0.002028	0.000662
Human Diseases	Infectious diseases	05150 Staphylococcus aureus infection [PATH:ko05150]	0.030502	0.021524	0.010512	0.174058
Human Diseases	Neurodegenerative diseases	05010 Alzheimer's disease [PATH:ko05010]	0.000880	0.000132	0	0.000786
Human Diseases	Neurodegenerative diseases	05012 Parkinson's disease [PATH:ko05012]	0	0	0	0.000357
Human Diseases	Substance dependence	05030 Cocaine addiction [PATH:ko05030]	0.000119	0.000084	0	0
Metabolism	Amino acid metabolism	00250 Alanine, aspartate and glutamate metabolism [PATH:ko00250]	5.524145	5.492114	5.272254	5.289959
Metabolism	Amino acid metabolism	00260 Glycine, serine and threonine metabolism [PATH:ko00260]	3.244323	2.912098	2.994151	3.031144
Metabolism	Amino acid metabolism	00270 Cysteine and methionine metabolism [PATH:ko00270]	2.104482	2.600167	2.500262	2.083621
Metabolism	Amino acid metabolism	00280 Valine, leucine and isoleucine degradation [PATH:ko00280]	0.707426	0.565010	0.528968	0.485956
Metabolism	Amino acid metabolism	00290 Valine, leucine and isoleucine biosynthesis [PATH:ko00290]	1.828326	2.063256	2.386016	1.846560
Metabolism	Amino acid metabolism	00300 Lysine biosynthesis [PATH:ko00300]	1.811626	2.070017	1.857568	1.935998

Metabolism	Amino acid metabolism	00310 Lysine degradation [PATH:ko00310]	0.045568	0.057816	0.089381	0.039451
Metabolism	Amino acid metabolism	00330 Arginine and proline metabolism [PATH:ko00330]	2.070231	2.110306	2.166754	1.707950
Metabolism	Amino acid metabolism	00340 Histidine metabolism [PATH:ko00340]	1.106824	1.194488	1.203264	0.912890
Metabolism	Amino acid metabolism	00350 Tyrosine metabolism [PATH:ko00350]	0.336808	0.393100	0.507080	0.680020
Metabolism	Amino acid metabolism	00360 Phenylalanine metabolism [PATH:ko00360]	0.388464	0.310979	0.338793	0.254572
Metabolism	Amino acid metabolism	00380 Tryptophan metabolism [PATH:ko00380]	0.036145	0.004032	0.000792	0.051820
Metabolism	Amino acid metabolism	00400 Phenylalanine, tyrosine and tryptophan biosynthesis [PATH:ko00400]	0.972707	0.747697	0.827996	0.835164
Metabolism	Biosynthesis of other secondary metabolites	00311 Penicillin and cephalosporin biosynthesis [PATH:ko00311]	0	0.000049	0	0
Metabolism	Biosynthesis of other secondary metabolites	00312 beta-Lactam resistance [PATH:ko00312]	0.006798	0.002397	0.005494	0.006826
Metabolism	Biosynthesis of other secondary metabolites	00521 Streptomycin biosynthesis [PATH:ko00521]	0.952181	0.706120	1.070862	1.073504
Metabolism	Biosynthesis of other secondary metabolites	00940 Phenylpropanoid biosynthesis [PATH:ko00940]	1.013448	0.594014	0.645047	1.039915
Metabolism	Biosynthesis of Other Secondary Metabolites	00941 Flavonoid biosynthesis [PATH:ko00941]	0.000098	0	0	0
Metabolism	Biosynthesis of other secondary metabolites	00945 Stilbenoid, diarylheptanoid and gingerol biosynthesis [PATH:ko00945]	0	0.000330	0	0.008702
Metabolism	Biosynthesis of other secondary metabolites	00960 Tropane, piperidine and pyridine alkaloid biosynthesis [PATH:ko00960]	0.000119	0.013373	0.004395	0.000689
Metabolism	Carbohydrate metabolism	00010 Glycolysis / Gluconeogenesis [PATH:ko00010]	2.852132	3.309255	2.638758	2.348353
Metabolism	Carbohydrate metabolism	00020 Citrate cycle (TCA cycle) [PATH:ko00020]	1.082470	0.511898	0.590755	0.841216
Metabolism	Carbohydrate metabolism	00030 Pentose phosphate pathway [PATH:ko00030]	1.247451	1.480809	1.361495	1.832354
Metabolism	Carbohydrate metabolism	00040 Pentose and glucuronate interconversions [PATH:ko00040]	1.525952	1.462787	1.476369	1.461933
Metabolism	Carbohydrate metabolism	00051 Fructose and mannose metabolism [PATH:ko00051]	1.198825	0.555040	0.695164	1.098311
Metabolism	Carbohydrate metabolism	00052 Galactose metabolism [PATH:ko00052]	2.207629	2.268026	2.050028	1.954769
Metabolism	Carbohydrate metabolism	00053 Ascorbate and aldarate metabolism [PATH:ko00053]	0.030795	0.021303	0.027339	0.020507
Metabolism	Carbohydrate metabolism	00500 Starch and sucrose metabolism [PATH:ko00500]	1.924107	2.288293	2.391785	2.312768
Metabolism	Carbohydrate metabolism	00520 Amino sugar and nucleotide sugar metabolism [PATH:ko00520]	1.690948	1.733578	1.788720	1.639225
Metabolism	Carbohydrate metabolism	00562 Inositol phosphate metabolism [PATH:ko00562]	0.033163	0.102913	0.125676	0.097285
Metabolism	Carbohydrate metabolism	00620 Pyruvate metabolism [PATH:ko00620]	2.273406	2.849409	2.508084	2.718437

Metabolism	Carbohydrate metabolism	00630 Glyoxylate and dicarboxylate metabolism [PATH:ko00630]	0.173743	0.226558	0.227961	0.255772
Metabolism	Carbohydrate metabolism	00640 Propanoate metabolism [PATH:ko00640]	0.043013	0.040584	0.055955	0.011718
Metabolism	Carbohydrate metabolism	00650 Butanoate metabolism [PATH:ko00650]	0.248851	0.321416	0.154792	0.151530
Metabolism	Carbohydrate metabolism	00660 C5-Branched dibasic acid metabolism [PATH:ko00660]	0.009524	0.081466	0.032062	0.033815
Metabolism	Energy metabolism	00190 Oxidative phosphorylation [PATH:ko00190]	2.495418	1.915229	2.381371	2.329239
Metabolism	Energy metabolism	00680 Methane metabolism [PATH:ko00680]	0.394826	0.720278	0.621958	0.689541
Metabolism	Energy metabolism	00710 Carbon fixation in photosynthetic organisms [PATH:ko00710]	0.019974	0.010729	0.072322	0.117446
Metabolism	Energy metabolism	00720 Carbon fixation pathways in prokaryotes [PATH:ko00720]	0.374747	0.431598	0.536224	0.482650
Metabolism	Energy metabolism	00910 Nitrogen metabolism [PATH:ko00910]	0.032092	0.025711	0.054164	0.021254
Metabolism	Energy metabolism	00920 Sulfur metabolism [PATH:ko00920]	0.136941	0.009220	0.009333	0.014865
Metabolism	Glycan biosynthesis and metabolism	00510 N-Glycan biosynthesis [PATH:ko00510]	0.026256	0.000698	0.003936	0.011822
Metabolism	Glycan biosynthesis and metabolism	00511 Other glycan degradation [PATH:ko00511]	0.360034	0.153645	0.084772	0.069591
Metabolism	Glycan biosynthesis and metabolism	00512 Mucin type O-glycan biosynthesis [PATH:ko00512]	0	0.000049	0	0
Metabolism	Glycan biosynthesis and metabolism	00531 Glycosaminoglycan degradation [PATH:ko00531]	0.044563	0.000225	0.020320	0.001726
Metabolism	Glycan biosynthesis and metabolism	00532 Glycosaminoglycan biosynthesis - chondroitin sulfate / dermatan sulfate [PATH:ko00532]	0	0.000142	0	0
Metabolism	Glycan biosynthesis and metabolism	00533 Glycosaminoglycan biosynthesis - keratan sulfate [PATH:ko00533]	0	0.000142	0	0
Metabolism	Glycan biosynthesis and metabolism	00540 Lipopolysaccharide biosynthesis [PATH:ko00540]	0.350973	0.026929	0.067753	0.043795
Metabolism	Glycan biosynthesis and metabolism	00550 Peptidoglycan biosynthesis [PATH:ko00550]	1.203687	0.835101	1.037116	1.146043
Metabolism	Lipid metabolism	00061 Fatty acid biosynthesis [PATH:ko00061]	1.108761	0.825275	0.974762	1.245448
Metabolism	Lipid metabolism	00071 Fatty acid metabolism [PATH:ko00071]	0.000189	0.004896	0	0.002230
Metabolism	Lipid metabolism	00120 Primary bile acid biosynthesis [PATH:ko00120]	0.044686	0.019083	0.025081	0.054500
Metabolism	Lipid metabolism	00140 Steroid hormone biosynthesis [PATH:ko00140]	0.014420	0	0.002245	0.001382
Metabolism	Lipid metabolism	00561 Glycerolipid metabolism [PATH:ko00561]	0.388770	0.669935	0.546262	0.729009
Metabolism	Lipid metabolism	00564 Glycerophospholipid metabolism [PATH:ko00564]	0.477019	0.550714	0.486880	0.551189
Metabolism	Lipid metabolism	00590 Arachidonic acid metabolism [PATH:ko00590]	0.003353	0.006091	0.003949	0.010961

Metabolism	Lipid metabolism	01040 Biosynthesis of unsaturated fatty acids [PATH:ko01040]	0.000639	0.003742	0.054474	0.037598
Metabolism	Metabolism of cofactors and vitamins	00130 Ubiquinone and other terpenoid-quinone biosynthesis [PATH:ko00130]	0.306733	0.056191	0.145714	0.185938
Metabolism	Metabolism of cofactors and vitamins	00670 One carbon pool by folate [PATH:ko00670]	0.431615	0.430236	0.402261	0.364897
Metabolism	Metabolism of cofactors and vitamins	00730 Thiamine metabolism [PATH:ko00730]	0.680639	0.357630	0.601086	0.448361
Metabolism	Metabolism of cofactors and vitamins	00740 Riboflavin metabolism [PATH:ko00740]	0.250492	0.087781	0.090529	0.212203
Metabolism	Metabolism of cofactors and vitamins	00750 Vitamin B6 metabolism [PATH:ko00750]	0.246018	0.029086	0.059575	0.116727
Metabolism	Metabolism of cofactors and vitamins	00760 Nicotinate and nicotinamide metabolism [PATH:ko00760]	0.813018	0.574383	0.823517	0.717025
Metabolism	Metabolism of cofactors and vitamins	00770 Pantothenate and CoA biosynthesis [PATH:ko00770]	0.428412	0.274855	0.373278	0.341389
Metabolism	Metabolism of cofactors and vitamins	00780 Biotin metabolism [PATH:ko00780]	0.234450	0.044921	0.076317	0.130424
Metabolism	Metabolism of cofactors and vitamins	00785 Lipoic acid metabolism [PATH:ko00785]	0.038581	0.007648	0.027309	0.049699
Metabolism	Metabolism of cofactors and vitamins	00790 Folate biosynthesis [PATH:ko00790]	0.269459	0.164167	0.176740	0.132451
Metabolism	Metabolism of cofactors and vitamins	00860 Porphyrin and chlorophyll metabolism [PATH:ko00860]	0.668177	0.726352	0.510521	0.625926
Metabolism	Metabolism of other amino acids	00430 Taurine and hypotaurine metabolism [PATH:ko00430]	0	0	0	0.001260
Metabolism	Metabolism of other amino acids	00440 Phosphonate and phosphinate metabolism [PATH:ko00440]	0.115392	0.089151	0.085840	0.167832
Metabolism	Metabolism of other amino acids	00450 Selenocompound metabolism [PATH:ko00450]	0.079972	0.110047	0.188971	0.180503
Metabolism	Metabolism of other amino acids	00480 Glutathione metabolism [PATH:ko00480]	0.218961	0.069748	0.252620	0.365016
Metabolism	Metabolism of terpenoids and polyketides	00281 Geraniol degradation [PATH:ko00281]	0.160166	0.003786	0.016464	0.010862
Metabolism	Metabolism of terpenoids and polyketides	00900 Terpenoid backbone biosynthesis [PATH:ko00900]	0.832114	0.711969	0.968719	0.603458
Metabolism	Metabolism of terpenoids and polyketides	00906 Carotenoid biosynthesis [PATH:ko00906]	0	0	0	0.000345
Metabolism	Metabolism of terpenoids and polyketides	00908 Zeatin biosynthesis [PATH:ko00908]	0.118197	0.058172	0.113136	0.066590
Metabolism	Metabolism of terpenoids and polyketides	01053 Biosynthesis of siderophore group nonribosomal peptides [PATH:ko01053]	0.002604	0.010330	0.025601	0.008675

Metabolism	Nucleotide metabolism	00230 Purine metabolism [PATH:ko00230]	3.829498	4.011497	4.095640	3.850074
Metabolism	Nucleotide metabolism	00240 Pyrimidine metabolism [PATH:ko00240]	1.797507	1.945831	1.854745	1.770959
Metabolism	Xenobiotics biodegradation and metabolism	00361 Chlorocyclohexane and chlorobenzene degradation [PATH:ko00361]	0.000274	0.003926	0.003930	0.004264
Metabolism	Xenobiotics biodegradation and metabolism	00362 Benzoate degradation [PATH:ko00362]	0.034285	0.001111	0.000409	0.005844
Metabolism	Xenobiotics biodegradation and metabolism	00622 Xylene degradation [PATH:ko00622]	0	0.000318	0	0
Metabolism	Xenobiotics Biodegradation and Metabolism	00624 Polycyclic aromatic hydrocarbon degradation [PATH:ko00624]	0	0.000071	0	0
Metabolism	Xenobiotics biodegradation and metabolism	00625 Chloroalkane and chloroalkene degradation [PATH:ko00625]	0	0.000587	0.000200	0
Metabolism	Xenobiotics biodegradation and metabolism	00627 Aminobenzoate degradation [PATH:ko00627]	0.001145	0.003898	0.001649	0.016919
Metabolism	Xenobiotics biodegradation and metabolism	00633 Nitrotoluene degradation [PATH:ko00633]	0.018802	0.005946	0.004589	0.033606
Metabolism	Xenobiotics biodegradation and metabolism	00643 Styrene degradation [PATH:ko00643]	0.000584	0.000049	0	0.000228
Metabolism	Xenobiotics biodegradation and metabolism	00791 Atrazine degradation [PATH:ko00791]	0.002139	0.015443	0.004546	0.011467
Metabolism	Xenobiotics biodegradation and metabolism	00930 Caprolactam degradation [PATH:ko00930]	0	0.000049	0	0
Metabolism	Xenobiotics biodegradation and metabolism	00983 Drug metabolism - other enzymes [PATH:ko00983]	0.041978	0.032385	0.025162	0.006019
Organismal Systems	Circulatory system	04260 Cardiac muscle contraction [PATH:ko04260]	0	0	0	0.000087
Organismal Systems	Development	04360 Axon guidance [PATH:ko04360]	0	0	0.000405	0
Organismal Systems	Digestive system	04974 Protein digestion and absorption [PATH:ko04974]	0.241116	0	0.047391	0.053256
Organismal Systems	Endocrine system	04910 Insulin signaling pathway [PATH:ko04910]	0.015526	0.004160	0.009230	0.000877
Organismal Systems	Environmental adaptation	04626 Plant-pathogen interaction [PATH:ko04626]	0.550139	0.508011	0.368556	0.355691
Organismal Systems	Excretory system	04962 Vasopressin-regulated water reabsorption [PATH:ko04962]	0	0.000147	0	0

Table S13: **Average relative abundance of functional genes, with significantly different relative abundance between control and H57 fed chickens, at level 3 SEED subsystem classification in the caecum of chickens fed sorghum based and wheat based diet at the age of day 13**

Functional genes (SEED Subsystem Level 3 Classification)	Average Relative Abundance					
	Sorghum Control	sorghum H57	P-value*	Wheat Control	Wheat H57	P-value*
At5g63420	0.160712	0.254225	0.015	0.219713	0.372993	0.002
Dihydroxyacetone_kinases	0.017816	0.050920	0.002	0.002339	0.055145	0.022
Macromolecular_synthesis_operon	0.385415	0.683395	0.008	0.707608	0.411347	0.042
Mannose_Metabolism	0.634260	0.132016	0.028	0.067116	0.256118	0.019
Translation_termination_factors_bacterial	0.255279	0.340528	0.034	0.252377	0.359311	0.029
tRNA_aminoacylation,_Ile	0.089506	0.204248	0.018	0.139186	0.291252	0.041
16S_rRNA_modification_within_P_site_of_ribosome	0.084276	0.120724	0.048	0.089017	0.182569	0.076
ABC_transporter_oligopeptide_(TC_3.A.1.5.1)	0.253477	0.562275	0.031	0.484378	0.752073	0.127
Aerotolerance_operon_in_Bacteroides_and_potentially_orthologous_operons_in_other_organisms	0.186869	0.000000	0.028	0.027017	0.023013	0.898
Bacterial_Cytoskeleton	0.762569	1.072622	0.019	0.798786	1.088408	0.177
CBSS-176280.1.peg.1561	0.213466	0.048551	0.023	0.133435	0.068360	0.302
CBSS-176299.4.peg.1292	0.185379	0.050513	0.006	0.102200	0.062707	0.135
CBSS-194948.1.peg.143	0.040786	0.013504	0.022	0.007168	0.016421	0.130
CBSS-288000.5.peg.1793	0.009607	0.107125	0.019	0.039475	0.065211	0.523
CBSS-323850.3.peg.3284	0.056356	0.000000	0.042	0.000000	0.000302	0.347
CBSS-354.1.peg.2917	0.073085	0.006676	0.047	0.047666	0.047300	0.989
CBSS-56780.10.peg.1536	0.149081	0.091014	0.010	0.073100	0.092464	0.553
CBSS-84588.1.peg.1247	0.024861	0.047254	0.048	0.047217	0.036972	0.691
COG1836	0.182863	0.102837	0.037	0.138256	0.100169	0.371
COG4319	0.041987	0.000540	0.036	0.010410	0.004138	0.306
Cluster_with_phosphopentomutase_paralog	0.013086	0.062627	0.006	0.011907	0.036557	0.302
Colanic_acid_biosynthesis	0.131809	0.004608	0.015	0.041376	0.005768	0.303
Cysteine_Biosynthesis	0.347691	0.178312	0.026	0.178091	0.150620	0.753
D-Sorbitol(D-Glucitol)_and_L-Sorbose_Utilization	0.005202	0.014528	0.034	0.025652	0.052077	0.162
DNA_gyrase_subunits	0.229997	0.445423	0.007	0.378770	0.307906	0.398

DNA_processing_cluster	0.244496	0.461746	0.032	0.310657	0.237166	0.578
Dipeptidases_(EC_3.4.13.-)	0.071775	0.018199	0.019	0.026321	0.022120	0.794
Dipicolinate_Synthesis	0.032358	0.073382	0.006	0.071149	0.022880	0.073
EC49-61	0.087293	0.014622	0.013	0.032776	0.029589	0.858
Extracellular_Polysaccharide_Biosynthesis_of_Streptococci	0.001897	0.010004	0.033	0.009191	0.005693	0.585
Fatty_Acid_Biosynthesis_FASII	0.635238	0.736901	0.021	0.661999	0.724958	0.565
Fatty_acid_metabolism_cluster	0.081068	0.002885	0.036	0.061147	0.066833	0.882
Glutamate_dehydrogenases	0.084460	0.240150	0.022	0.207457	0.264670	0.636
Glutathione:_Non-redox_reactions	0.020414	0.000623	0.037	0.000222	0.002643	0.073
Glycine_and_Serine_Utilization	0.321013	0.171071	0.031	0.243909	0.315215	0.439
Heat_shock_dnaK_gene_cluster_extended	0.419260	0.642104	0.014	0.413703	0.529957	0.079
Heme,_hemin_uptake_and_utilization_systems_in_GramNegatives	0.038658	0.002058	0.019	0.008741	0.006995	0.841
Iron_acquisition_in_Vibrio	0.509186	0.021723	0.016	0.236633	0.046111	0.407
Lactose_and_Galactose_Uptake_and_Utilization	0.820842	0.555635	0.015	0.674726	0.632842	0.620
Lipopolysaccharide_assembly	0.152870	0.012780	0.033	0.008895	0.022403	0.080
Listeria_phi-A118-like_prophages	0.002511	0.031147	0.020	0.025452	0.008546	0.284
Mebrane_bound_hydrogenases	0.011544	0.067829	0.028	0.101739	0.009192	0.062
Multidrug_Resistance_Efflux_Pumps	0.592731	0.182356	0.013	0.071999	0.132943	0.186
Na(+)-translocating_NADH-quinone_oxidoreductase_and_rnf-like_group_of_electron_transport_complexes	0.384430	0.191682	0.025	0.145238	0.127350	0.618
Neotrehalosadiamine_(NTD)_Biosynthesis_Operon	0.000000	0.000284	0.049	0.000202	0.000000	0.347
P_uptake_(cyanobacteria)	0.205530	0.090542	0.038	0.134285	0.117538	0.703
Peptidyl-prolyl_cis-trans_isomerase	0.058016	0.002726	0.037	0.020962	0.004579	0.406
Periplasmic_disulfide_interchange	0.059301	0.000000	0.006	0.014955	0.002806	0.429
Perosamine_Synthesis_Vibrio	0.055748	0.001688	0.048	0.025321	0.015860	0.532
Photorespiration_(oxidative_C2_cycle)	0.122651	0.032328	0.003	0.062594	0.094831	0.054
Potassium_homeostasis	0.355791	0.121590	0.034	0.342436	0.292794	0.659
Protein_degradation	0.392461	0.093878	0.046	0.123670	0.212990	0.076
Protocatechuate_branch_of_beta-ketoadipate_pathway	0.013126	0.076594	0.029	0.023240	0.048976	0.424
Pterin_metabolism_3	0.040383	0.003303	0.040	0.012976	0.012081	0.917
Pyridoxin_(Vitamin_B6)_Biosynthesis	0.320265	0.240357	0.007	0.187451	0.254887	0.249
Respiratory_Complex_I	0.381903	0.008948	0.027	0.039978	0.015003	0.505

Serine-glyoxylate_cycle	1.045412	0.498338	0.013	0.569767	0.561567	0.886
Sporulation-related_Hypotheticals	0.096656	0.212102	0.026	0.173643	0.241839	0.239
Sporulation_Cluster	0.059317	0.163338	0.044	0.074200	0.076846	0.954
Streptococcal_Mga_Regulon	0.076353	0.016151	0.029	0.007241	0.019610	0.284
The_fimbrial_Stf_cluster	0.000105	0.000595	0.042	0.000000	0.003318	0.072
The_mdtABCD_multidrug_resistance_cluster	0.198830	0.014779	0.040	0.021320	0.006180	0.489
Threonine_and_Homoserine_Biosynthesis	0.351356	0.193797	0.028	0.240641	0.348940	0.248
Ton_and_Tol_transport_systems	0.534177	0.003841	0.019	0.218429	0.042522	0.415
Transport_of_Zinc	0.074171	0.010992	0.034	0.038398	0.048150	0.727
Trehalose_Uptake_and_Utilization	0.271206	0.769278	0.009	0.571990	0.459153	0.627
polyprenyl_synthesis	0.072882	0.129949	0.027	0.156188	0.082952	0.147
r1t-like_streptococcal_phages	0.462801	0.811922	0.026	0.588664	0.512730	0.770
rRNAs	0.056121	0.111901	0.000	0.067386	0.081141	0.673
tRNA_aminoacylation,_Arg	0.145788	0.052261	0.002	0.079855	0.192663	0.204
tRNA_aminoacylation,_Tyr	0.134038	0.037786	0.013	0.071090	0.112977	0.403
Alkylphosphonate_utilization	0.015762	0.018564	0.866	0.002890	0.017093	0.035
Biogenesis_of_c-type_cytochromes	0.027314	0.004958	0.344	0.002220	0.008717	0.043
CBSS-176279.3.peg.868	0.027273	0.031411	0.764	0.041222	0.093297	0.043
CBSS-323850.3.peg.3269	0.062387	0.112535	0.066	0.170043	0.045682	0.022
Conjugative_transfer_related_cluster	0.064287	0.125281	0.126	0.173445	0.046459	0.045
DNA-binding_regulatory_proteins,_strays	0.004312	0.010490	0.320	0.003094	0.021756	0.041
Fermentations:_Lactate	0.120414	0.213535	0.312	0.238872	0.484384	0.028
Fermentations:_Mixed_acid	0.252937	0.411548	0.107	0.538207	0.867814	0.004
Formate_hydrogenase	0.030912	0.059308	0.338	0.008054	0.068106	0.034
Galactose-inducible_PTS	0.012950	0.015466	0.823	0.011640	0.054189	0.048
Glutamate_and_Aspartate_uptake_in_Bacteria	0.008906	0.011084	0.768	0.006962	0.044260	0.022
Mannitol_Utilization	0.040250	0.039273	0.966	0.058238	0.143277	0.007
Molybdopterin_cytosine_dinucleotide	0.000482	0.003315	0.378	0.000000	0.001438	0.012
Multiple_Antibiotic_Resistance_MAR_locus	0.000000	0.000054	0.347	0.000000	0.001094	0.040
Murein_Hydrolases	0.054492	0.038688	0.672	0.027947	0.068396	0.029
Pentose_phosphate_pathway	0.245411	0.335315	0.178	0.269508	0.531579	0.038

Periplasmic_Stress_Response	0.003793	0.002698	0.742	0.000417	0.010278	0.012
Phage_regulation_of_gene_expression	0.039503	0.023652	0.442	0.015377	0.050165	0.042
Purine_Utilization	0.113932	0.212878	0.162	0.141607	0.321402	0.050
RNA_pseudouridine_syntheses	0.042751	0.050298	0.594	0.037288	0.100224	0.020
Respiratory_dehydrogenases_1	0.064623	0.024315	0.221	0.008686	0.069792	0.017
Toxin-antitoxin_systems_(other_than_RelBE_and_MazEF)	0.028845	0.046654	0.249	0.055145	0.013832	0.039
Transcription_factors_bacterial	0.270726	0.207807	0.438	0.177510	0.312108	0.027
Type_1_pili_(mannose-sensitive_fimbriae,_gamma-fimbriae)	0.000791	0.000818	0.976	0.000596	0.004062	0.006
Wyeosine-MimG_Biosynthesis	0.028776	0.012880	0.128	0.009121	0.072601	0.002
YggW	0.024045	0.022968	0.924	0.016868	0.037697	0.035
tRNAmodification_position_34	0.066658	0.104169	0.187	0.076767	0.124333	0.044
trimethylamine_N-oxide_(TMAO)_reductase	0.000162	0.007294	0.189	0.000202	0.005394	0.041
*Highlighted cells indicate statistically significant						

Table S14: **Average relative abundance of genes encoding KEGG Orthology (KO) level 1 functions in the caecum of chickens fed sorghum based and wheat based diet at the age of day 13**

KO Level 1	Average Relative Abundance			
	Sorghum		Wheat	
	Control	H57	Control	H57
Cellular Processes	3.48	2.99	3.28	2.67
Environmental Information Processing	11.66	14.53	14.66	14.87
Genetic Information Processing	25.23	26.11	24.44	24.81
Metabolism	57.79	55.13	56.44	56.05
Human Diseases	1.02	0.73	0.76	1.18
Organismal Systems	0.81	0.51	0.43	0.41

Table S15: **Average relative abundance of genes encoding SEED subsystem level 1 functions in the caecum of chickens fed sorghum based and wheat based diet at the age of day 13**

Subsystem level 1	Average Relative Abundance			
	Sorghum		Wheat	
	Control	H57	Control	H57
Carbohydrates	17.56	18.73	17.39	17.65
Clustering-based subsystems	12.34	13.15	13.42	12.70
Protein Metabolism	9.98	10.04	9.76	10.61
Amino Acids and Derivatives	8.88	8.75	9.68	9.61
DNA Metabolism	6.93	7.81	7.05	6.43
RNA Metabolism	5.14	5.18	5.10	5.54
Miscellaneous	4.66	4.41	5.05	5.15
Phages, Prophages, Transposable elements, Plasmids	4.54	3.20	3.11	3.06
Cell Wall and Capsule	4.07	2.67	3.54	3.33
Cofactors, Vitamins, Prosthetic Groups, Pigments	3.78	2.98	3.32	3.27
Nucleosides and Nucleotides	3.50	3.80	3.99	4.20
Virulence, Disease and Defense	3.04	2.36	1.94	1.77
Membrane Transport	2.86	3.30	3.29	3.53
Respiration	2.86	2.97	2.84	3.29
Fatty Acids, Lipids, and Isoprenoids	1.47	1.48	1.76	1.94
Cell Division and Cell Cycle	1.39	2.14	1.85	1.83
Stress Response	1.22	1.62	1.18	1.25
Phosphorus Metabolism	1.22	1.03	1.39	1.09
Iron acquisition and metabolism	1.12	0.80	0.86	0.50
Regulation and Cell signaling	0.80	0.61	0.64	0.78
Nitrogen Metabolism	0.71	0.78	0.69	0.61
Sulfur Metabolism	0.62	0.57	0.68	0.38
Dormancy and Sporulation	0.40	0.79	0.53	0.44
Potassium metabolism	0.39	0.14	0.40	0.34
Metabolism of Aromatic Compounds	0.31	0.44	0.32	0.44
Motility and Chemotaxis	0.15	0.14	0.10	0.14
Photosynthesis	0.03	0.00	0.00	0.01
Secondary Metabolism	0.03	0.09	0.09	0.10

Appendix 4. Animal Ethics Approval Certificate

THE UNIVERSITY OF QUEENSLAND
AUSTRALIA

UQ Research and Innovation
Director, Research Management Office
Nicole Thompson

Animal Ethics Approval Certificate

23-Jun-2016

Please check all details below and inform the Animal Welfare Unit within 10 working days if anything is incorrect.

Activity Details

Chief Investigator:	Professor Wayne Bryden, Animal Science
Title:	Evaluation of the probiotic, H57 in poultry nutrition studies
AEC Approval Number:	SAFS/159/16/ARC
Previous AEC Number:	SAFS/086/13/ARC
Approval Duration:	24-Jun-2016 to 24-Jun-2019
Funding Body:	ARC
Group:	Production and Companion Animal
Other Staff/Students:	Xiuhua Li, Dagong Zhang
Location(s):	Gatton Bldg 8258 - Poultry Research Unit

Summary

Subspecies	Strain	Class	Gender	Source	Approved	Remaining
Poultry	Broiler	Juvenile / Weaners / Pouch animal	Male	Commercial breeding colony	1200	1200

Permits

Provisos

Logbook Proviso:
The CI is required to keep a logbook of all students who will be participating in this protocol, with this information provided to the AEC as required.

Approval Details

Description	Amount	Balance
Poultry (Broiler, Male, Juvenile / Weaners / Pouch animal, Commercial breeding colony)		
15 Jun 2016 Initial approval	1200	1200

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